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144. INVESTIGATIONS INTO THE CHEMISTRY OF THE FLUE-CURING OF TOBACCO

By E. A. HOUGHTON ROBERTS

From the Tocklai Experimental Station, Indian Tea Association, Cinnamara P.O., Assam

(*Received 5 December 1941*)

In connexion with an attempt to grow in Assam tobacco suitable for the manufacture of cigarettes, the opportunity was taken of starting investigations upon the chemical changes that occur in flue-curing. This appears to be the process best suited for the type of leaf grown.

The curing was carried out in substantially the same way as recommended by Brown [1933]. The curing barn is filled with leaf, preferably of the same texture and ripeness, and the barn is then sealed. The door and all ventilators are closed, so that no moisture can escape. The furnace is lit and the temperature of the barn maintained at 85–90° F. At this temperature metabolic processes will be taking place almost at their maximum rate and the sealing of the barn ensures that the loss of moisture will be slight. The temperature is maintained at about 90° F. until the leaf begins to yellow at the edges, after which the temperature is slowly raised, 5° at a time, still keeping the barn sealed. During this period the yellow colour spreads over the leaf and by the time the temperature has reached 110° F. the whole leaf should be yellow, with only a slight greenish tinge. The full yellow colour should be developed by the time the temperature has reached 115° F. The time taken to reach this stage will depend upon the nature of the leaf. In our experiments it took from 36 to 48 hr. Meanwhile it is necessary to reduce the humidity in the barn somewhat. If the humidity remains high with the temperature above 110° F. the leaf will assume a reddish brown colour. This defect is generally referred to as sponging, and it is believed that we can now account for this, as will be shown later in this paper. On the other hand if the leaf is allowed to dry out too rapidly the cells are killed prematurely and the chemical changes characteristic of curing are arrested. The leaf then remains green in patches.

To fix the bright lemon-yellow colour it is necessary to strike a mean between these two courses. The humidity in the barn is reduced by opening the ventilators gradually. Fig. 1 shows the stages at which humidity was increased in one curing. The ventilators were quarter opened when the temperature reached 105° and ventilation was then gradually increased up to 120° F. when the leaf began to curl. At 135–140°, when the ventilators are fully open, the blade of the leaf is practically dried out, but the midrib still remains moist. This is dried out by increasing the temperature in stages to 160°, during which period the ventilation is again reduced. This temperature also serves largely to inactivate the enzymes so that when the cured leaf is allowed to take up moisture again (for greater ease of handling without damage) further chemical changes in the tissue are arrested.

The chemical changes that occur during flue-curing do not appear to have been fully investigated. Vickery *et al.* [1933] have studied the curing to the brown stage of Connecticut shade-grown tobacco, and other investigations on

miscellaneous curing processes have been summarized by Bodnar & Barta [1935]. These changes bear a strong resemblance to those found to occur during water culture in the dark of the tobacco leaf.

These latter changes have been studied in great detail by Vickery *et al.* [1937], and Chibnall [1939] has summarized these findings. An outline of the conclusions is given below.

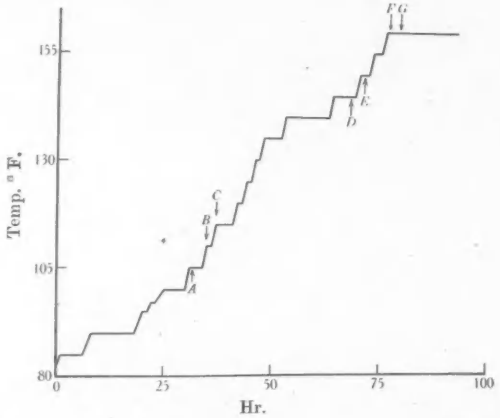

Fig. 1. *A*, top and bottom ventilators ¼ open. *B* top and bottom ventilators ½ open. *C*, top and bottom ventilators fully open. *D*, bottom ventilator ½ closed. *E*, bottom ventilator fully closed. *F*, top ventilator ½ closed. *G*, top ventilator fully closed.

Tobacco leaves were detached from the plant early in the morning when carbohydrate content was at a minimum. As water culture was in the dark no further production of carbohydrate by photosynthesis occurred. The first 20 hr. of water culture Chibnall regards as the 'carbohydrate phase', for the carbohydrates are here the main source of energy. The organic acids are also concerned to some extent in the metabolic changes, while protein is hydrolysed to amino-acid and thence oxidized to NH_3, the latter condensing with fumaric or oxaloacetic acids to produce asparagine.

In the second phase, lasting from 20 to 70 hr., carbohydrates play a minor part, while significant changes take place in the organic acids. Protein decomposition and asparagine formation continue.

In the third and final phase lasting from the 70th hr. onwards the supply of organic acids has become exhausted and the leaf tissue now derives its energy almost entirely from protein. The leaves are yellowing, and disintegration of the chloroplasts and breakdown of the cytoplasm are increasing. In this 'protein phase' the amino-acids are eventually no longer able to function both as respiratory substrates and NH_3 acceptors (asparagine formation) and the resultant accumulation of NH_3 is probably the chief cause of the death of the cell.

The three phases might be expected to be characterized by R.Q. of 1, >1, and <1, but owing to overlapping of organic acid and protein phases the rise in R.Q. during the second phase may be obscured.

Experimental

Methods

The respiratory activity of tobacco leaf was measured essentially as described by Deb & Roberts [1940] for tea, using the Barcroft-Warburg apparatus [Dixon, 1934]. 300 mg. tobacco leaf in circles of area 1 cm.^{2} were weighed into each of four or five Warburg vessels and covered with 3 ml. $M/15$ phosphate buffer at pH 5·8. The inner cup contained a small roll of filter paper soaked with KOH. A second identical series of vessels was set up but in this case the KOH was omitted. After equilibration of temperature the manometric levels were read at suitable intervals over a period of 1–3 hr. O_2 uptakes are obtained directly from the vessels containing KOH. In the vessels without KOH, O_2 will be consumed and CO_2 given out, so that the net change in the manometric level will be considerably smaller. After making allowance for the difference in solubility of O_2 and CO_2 in water the CO_2 output can be calculated from the change in manometric levels of the vessels without KOH, and the observed O_2 uptake.

For tea the above method has proved accurate provided that care is taken in the sampling of the tissue, and although cumbrous, has the advantage over the Dickens & Šimer method of affording a measure of R.Q. at frequent intervals over a long period. In the Dickens & Šimer method the mean R.Q. over a given period may be determined with greater accuracy but no information is afforded as to the variations of the R.Q. within the experimental period.

CO_2 retention. The above method of estimating R.Q. has been of little use in working with animal tissues owing to the retention of CO_2 by phosphate buffers and protein. For plant tissues this CO_2 retention is of smaller importance. Turner [1938] finds no difference between the R.Q. of carrot tissue determined by this method and that of Dickens & Šimer, and my own observations with tea leaf show that CO_2 retention both at the beginning and end of a long period of shaking is entirely negligible.

With tobacco leaf CO_2 retention is appreciable. The amount of bound CO_2 in the tissue may be determined manometrically by tipping 0·5 ml. 2N H_2SO_4 from a side arm into the main compartment of the Warburg vessel which contains the tissue suspended in the buffer solution, thus liberating bound CO_2.

The following example, in which the R.Q. of leaf in the closing stages of curing is being determined, illustrates the magnitude of the retention effect. After 2 hr. shaking at 36°, the O_2 uptake per 100 mg. of tissue (moisture content 54·35 %) amounted to 194 μl. while the CO_2 output was 139 μl. This gives an average R.Q. of 0·72, uncorrected for CO_2 retention.

The CO_2 liberated on adding acid to the original tissue amounted to 48 μl. per 100 mg. tissue, while the amount set free after 2 hr. shaking was only 20 μl. It follows that 28 μl. (the difference between the above figures) of the bound CO_2 in the original tissue was set free in the course of 2 hr shaking, and this figure must be subtracted from the observed CO_2 output to give the amount of CO_2 produced in the course of metabolism. The CO_2 produced in the 2 hr. shaking thus amounts not to 139 μl. but 111 μl. and the R.Q. must be reduced from 0·72 to 0·57.

The correction is relatively small for fresh uncured leaf but increases as the process continues. As the leaf dries out, its CO_2 retention appears to increase. The evolution of the bound CO_2 during the shaking proceeds at a fairly uniform rate. For this reason the R.Q. values recorded over successive intervals of time, although too high, have considerable value in indicating the nature of the chemical changes taking place at different periods during the shaking.

O_2 tension. The R.Q. of young tobacco leaves has been measured on many occasions and does not depart significantly from 1·0. On the other hand the R.Q., as measured by the above method, of leaf ripe for curing varies from 1·3 to 1·7. It was at first thought that this implied a predominantly organic acid type of metabolism in such leaves but subsequent analyses showed that the leaf contained appreciable amounts of reducing sugars.

The ripe leaf is considerably thicker than the young leaves, for which an R.Q. of 1·0 was obtained, and the possibility arose that the high R.Q. was due to the inability of O_2 to diffuse into the interior of the tissue and to maintain an O_2 tension sufficient to ensure aerobic oxidation of the carbohydrates. In other words some anaerobic fermentation may also be taking place within the tissue with the result that the R.Q. would exceed unity.

The correctness of this view was shown by carrying out the determinations in an atmosphere of O_2 when the R.Q. value was found to be unity.

Table 1. *Apparent* R.Q. *in air and* O_2

Atmosphere	O_2 uptake/1 hr./ 100 mg. tissue	CO_2 output/1 hr./ 100 mg. tissue	R.Q.
Air	40	50	1·25
O_2	76	77	1·01

Results

Ripe tobacco leaf was only available for a comparatively short period and in limited amounts. The results obtained therefore are of a preliminary nature only but it is believed that the methods developed are suitable for a thorough investigation of the flue-curing process and it is hoped at a later date to carry out such an investigation. Meanwhile the preliminary results are presented in the hope that they may be of some assistance to other workers in the field.

We had neither the resources nor the amount of material to carry out full analyses such as were undertaken by Vickery and Pucher. Samples of leaf were taken at suitable intervals during the whole curing process, but these samples were not sufficiently large to be representative for chemical analysis. From the results obtained a decrease in total organic solids is apparent as the result of curing, but the sampling error was too high to enable an accurate estimate of this loss to be made.

For the purposes of the experiment six leaves were selected for each sample. Disks of 1 cm.^{2} were punched in the blades of these leaves, avoiding the mid-rib. In the dried-out leaf it was impossible to draw such samples and the tissue was cut by scissors into squares of approximately 1 cm.^{2} The samples were then thoroughly bulked and subsamples weighed out into the Warburg vessels. The agreement between replicates was a guarantee of the accuracy of this second sampling but of course there is no certainty that the original sample was truly representative. However, as the leaves were chosen to approximate to the mean degree of curing and as the quantity we are interested in is a ratio and not an absolute quantity it is possible that smaller samples may suffice than if an analysis were to be carried out.

As the barn is kept sealed for the first 24 hr. no sample during that time could be taken. Table 2 gives a typical result for leaf after it has been in the curing barn for 40 hr.

Table 2. *40 hr. cured leaf*

hr. ...	0–½	½–1	1–1½	1½–2
O_2	50	43	41	37
CO_2	48	40	36	33
R.Q.	0·96	0·93	0·88	0·89

Uptakes and outputs are expressed in μl. per 100 mg. tissue (moisture content 70·5%). No correction is made in this case for CO_2 retention but with leaf at this stage the effect is small and would only reduce the R.Q. by two or three units in the second decimal place.

The rate for the first half hour is equivalent to 3·4 μl. O_2 per mg. dry wt. per hr. against 3·7 μl. O_2 for the original uncured leaf. There is appreciable loss in solid matter during 40 hr. possibly amounting to 10% of the total so that the actual fall in respiratory activity is rather greater than would appear from the above figures.

There can be no doubt from the above result, confirmed as it has been on several occasions, that protein is being metabolized as a respiratory substrate at this stage of the process although carbohydrate, organic acids, or both, still account for the greater part of the respiration.

In general a decrease both in the R.Q. and in the respiratory coefficient (Q_{O_2}) is at first observed as curing proceeds, but when the temperature of the barn exceeds 120° F. a fresh series of effects is noted when samples are investigated manometrically.

The average R.Q. over a period of 2 hr. is well below 0·7, the value for protein oxidation, even without making the correction for CO_2 retention, which is quite appreciable in such cases. The initial rate of O_2 uptake is high, but this falls rapidly in contrast to the slow decrease observed for fresh leaf or for samples withdrawn at lower temperature in the barn. As the rate of O_2 uptake falls the R.Q. rises and eventually after about 2 hr. may reach 0·8. The Q_{O_2} value is then only some 10% of the original value for the uncured leaf. In general the longer the leaf has been in the barn at temperatures over 120° F. the lower is the R.Q. for the first half hour of shaking. The leaf when placed in the Warburg vessel loses its bright lemon-yellow colour and takes on a brown tint while the reaction medium instead of remaining colourless becomes reddish brown. This solution gives all the reactions for a polyphenol.

A typical series of figures for O_2 and CO_2 in such a case is given in Table 3.

Table 3

hr. ...	0–½	½–1	1–1½	1½–2	2–2½
O_2	150	55	33	20	24
CO_2	47	26	18	16	21
R.Q.	0·31	0·47	0·55	0·80	0·88

Note the rapid fall in the rate of O_2 uptake in successive half hour periods and the corresponding rise in the R.Q.

Reddening of partly cured tobacco leaf after moistening. Although this very characteristic effect is always observed in tobacco leaf after the temperature of the barn has reached 120° F., there is no reason to believe that it forms part of the normal curing process. Properly flue-cured tobacco always has a bright lemon-yellow colour whereas this chemical change in the Warburg vessel is associated with the development of a dark brown colour. Again, judging by the figures in Table 3, it takes about 1½ hr. to complete the reaction associated with the low R.Q. after which the chemical changes in the leaf are probably mainly those of protein degradation and oxidation. On the other hand, leaf may be left in the curing barn for another 48 hr. after 120° F. is reached and still be lemon-yellow, yet it will still undergo this low R.Q. change when suspended in water.

The most probable explanation of this effect is that at *c.* 120° F. the cytoplasm of the tobacco leaf undergoes a rapid change in its permeability so that the polyphenols may diffuse into it. In the curing when this point is reached the tissue is dried out to such an extent that diffusion of the vacuole contents into the cytoplasm is prevented, but if the tissue be moistened, as it is in the Warburg vessel, free penetration of the cytoplasm by the polyphenols may now take place with consequent oxidation to brownish red pigments.

The evidence in favour of this view will now be considered. A sudden increase in the permeability of the protoplasmic membrane of plant tissue at a moderately elevated temperature has been established in at least two other cases and there is every reason to believe that this is a general phenomenon. Thus Sen [1928] has shown that the electrical resistance of the *Mimosa pudica pulvinus* decreases slowly with increasing temperature over the range 30–50°, but at 55° there is a very sharp drop in the resistance. Sen interprets this as due to the complete permeability of the protoplasm to vacuole solvents at this temperature so that the tissue as a whole will have a lower electrical resistance.

A second example of this is provided by the effect of heat on tea leaf. If the leaf is maintained at a temperature of 120° F. under conditions where moisture loss is slow, it gradually reddens and eventually the whole leaf assumes a uniform reddish brown colour analogous to that produced in tea-fermentation. Microscopic examination reveals that in the reddened leaf the polyphenols have penetrated into the cytoplasm where they have been oxidized to form the pigments characteristic of fermented tea.

It appears that at temperatures of 50–60° the protoplasmic membranes lose their powers of semi-permeability and the cytoplasm becomes freely permeable to all solutes of the vacuole. If the contents of the vacuole include oxidizable substrates, such as polyphenols, these substances will then become oxidized by the oxidase system of the tissue and pigmentation follows, hence the reddening of both tea and tobacco leaf under these conditions.

The oxidation of polyphenols during the reddening of tobacco leaf is indicated by the low R.Q. recorded during this phase. Such oxidations in general involve no CO_2 formation so that the R.Q. is nil. With simultaneous protein and polyphenol oxidation the R.Q. may be anything between zero and 0·7 and this is in fact observed. The part played by polyphenols in this stage is also indicated by the appearance in the suspending liquid of polyphenol oxidation products.

The tobacco leaf is known to contain rutin, the rhamnoglucoside of quercetin, and it has been shown by Neuberg & Kobel [1935] that this substance is transformed by tobacco enzymes into a soluble brown pigment. The question arises as to whether the amount of rutin, or other polyphenols, in the tobacco leaf is sufficient to account for the low R.Q. phase observed in the experiments on partially cured tobacco.

It is possible to arrive at an approximate measure of the polyphenol content of green leaf tissue by a manometric method. If the tissue is ground to a fine paste, its respiratory powers are almost completely suppressed [Roberts, 1941] and unless it contains some oxidizable substrate such as a polyphenol a suspension of this paste in water will consume O_2 at only a very low rate. On the other hand if polyphenols are present there will be a more or less vigorous uptake of O_2 until oxidation of these substances is complete. The oxidized polyphenols will in turn promote a secondary oxidation of carbohydrate and in consequence some CO_2 will be produced, and the oxidized polyphenol will be again reduced. The efficiency of the oxidized polyphenol as a H-acceptor in such processes is not high as much of it undergoes further changes, probably

by polymerisation, into pigments incapable of oxidizing carbohydrates. Eventually the whole of the polyphenol is converted into these pigments and carbohydrate oxidation ceases. The reactions involved are analogous to those in the 'fermentation' of tea [Roberts, 1941] and may be summarized.

$$\text{Polyphenol} \underset{\text{dehydrogenase} + 2H}{\overset{\text{oxidase} - 2H}{\rightleftharpoons}} o\text{-quinone} \rightarrow \text{condensed pigments.}$$

The O_2 uptakes and CO_2 outputs recorded for a suspension of tobacco leaf are plotted in Fig. 2. Assuming that the oxidation of carbohydrate follows the course $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$, the amount of O_2 consumed in carbohydrate oxidation in this case equals 22 μl. per 100 mg. so that the amount of O_2 required for complete oxidation of the polyphenols must be 68–22 μl. In other words the polyphenol content of the fresh tobacco leaf is equivalent to 460 μl. O_2 per g. Assuming that only 1 atom O is required for the oxidation of rutin this is equivalent to 2·3 % rutin (or 1·2 % quercetin) on the fresh weight of the tissue. If 2 atoms O are consumed in the oxidation of the polyphenol these percentages will be correspondingly lower.

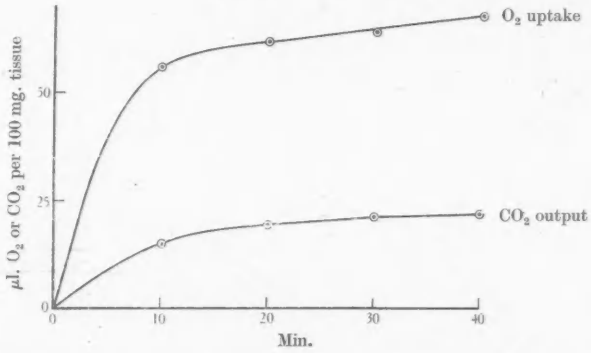

Fig. 2. O_2 uptake and CO_2 output of a suspension in water of finely ground tobacco leaf.

The polyphenol content of plant tissues may also be determined by another manometric method. The tobacco leaf is extracted thoroughly with boiling water and an aliquot of the infusion (2 ml.) placed in the main compartment of a Warburg vessel. After temperature equilibrium is reached 1 ml. 2*N* NaOH, is tipped in from a side arm and the O_2 uptake measured until the autoxidation has come to a standstill. Assuming that 5 atoms O are consumed per mol. polyphenol [Harrison & Roberts, 1939] the percentage polyphenol in the tobacco leaf, expressed as rutin, may be calculated from the uptake and the mol. wt. of rutin. On several samples of leaf the rutin content averaged 1·8 %, in fairly good agreement with the figure obtained by the earlier method, in view of the assumptions made.

We may now deal with the low R.Q. phase of the partly cured tobacco. In the example already quoted in Table 3 the O_2 uptake and CO_2 outputs after 2½ hr. amount to 282 and 128 μl. per 100 mg. tissue respectively. The CO_2 output must be corrected for the decrease in CO_2 retention of the tissue during this

period, and this reduces the figure for the true CO_2 output to 100 μl. This amount of CO_2 is produced by true respiratory processes and the R.Q. of the processes leading to CO_2 production at this stage of the curing may be assumed to be 0·8 without any serious error. This is equivalent to an uptake of 125 μl. O_2 and leaves the consumption of 157 μl. O_2 per 100 mg. tissue to be accounted for, possibly in terms of polyphenol oxidation.

In the original green leaf the O_2 equivalent of the polyphenols was 460 μl. O_2 per g. fresh leaf, or 2000 μl. O_2 per g. dry wt. For the partly cured leaf of moisture content 17 %, the O_2 equivalent unaccounted for is 1570 μl. per g. or 1890 μl. per g. dry wt. No account is here taken of the loss of dry wt. during the curing process but this loss is unlikely to exceed 15 % of the original dry matter of the leaf. Considering the assumptions made, it will be seen that the hypothesis that the low R.Q. phase is due to simultaneous oxidation of polyphenols and protein in the curing leaf is fully borne out by the correspondence between the polyphenol contents found in the fresh leaf and deduced in the partly cured leaf from a study of the R.Q. values.

Discussion

From the foregoing results it is possible to form an idea of the changes that take place in flue-curing. During the yellowing stage there is a steady fall both in the rate of O_2 consumption by the tissue and in the R.Q., corresponding to a gradual exhaustion of respiratory substrates and an increasing call on the protein matter for metabolic purposes.

The quality of the fully cured tobacco may well depend upon the extent to which these changes take place and an investigation on these lines might give much insight into the factors necessary for high quality leaf.

The cause of red leaf is now clear. If the leaf is still moist when the critical temperature is reached at which the semi-permeability of the protoplasmic membranes breaks down, polyphenols diffuse into the cytoplasm where they are oxidized to form brownish red pigments. To avoid this the leaf must be sufficiently dried out by the time the critical temperature is reached to prevent diffusion of the vacuole contents into the cytoplasm. It must be the aim of further researches to determine how far the leaf must be dried to prevent this diffusion.

It seems probable that in the fixing stage chemical changes do not take place to any great extent owing to the dryness of the tissue. Enzymes, however, are still active and if the tissue is moistened, as when suspended in water in Warburg vessels, both protein and polyphenol oxidation can take place. Only after the temperature has been maintained at 160° F. for some time, during which period the mid-rib is dried out, is it safe to allow the tissue to pick up moisture, for by then the enzymes are sufficiently destroyed and further marked chemical changes in the leaf are unlikely.

The author is indebted to the Indian Tea Association for permission to publish these results. He also wishes to thank the Director, Mr P. H. Carpenter, for his interest in this work and Mr N. M. Macgregor who undertook the practical task of curing the tobacco.

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145. THE SPECIFICITY OF ARGINASE: ACTION UPON ARGININIC ACID

By ANDREW HUNTER and HUBERT EDMUND WOODWARD

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(*Received 8 December 1941*)

In such arginine derivatives as have hitherto been tested, an unmodified guanidino group has appeared to be a condition prerequisite for the action of liver arginase [Thomas *et al.* 1922; Felix *et al.* 1928; Felix & Schneider, 1938]. Until recently an unmodified carboxyl group was thought to be in this respect of equal importance; but according to Richards & Hellerman [1940] agmatine is not, as formerly asserted, completely resistant to the enzyme, although it contains no carboxyl group. As for the third functional group of arginine—the α-amino group—it has been known for many years that modifications obstruct, but do not always completely block, the attack of arginase [cf. Hunter, 1938]. More recently it has become evident that the amino group may even be dispensed with. Although to Felix *et al.* [1928] argininic acid, in which the α-amino group of arginine is replaced by hydroxyl, appeared to be quite unaffected by arginase, Calvery & Block [1934] observed it to yield (under conditions rather imperfectly specified) as much as 85% of the theoretically possible amount of urea. Felix & Müller [1936] and Felix & Schneider [1938] later agreed that argininic acid was indeed hydrolysed by arginase, although much more slowly than arginine. Meanwhile Hellerman & Perkins [1935] had stated that a 'highly specific' arginase preparation failed to catalyse the hydrolysis of argininic acid; but Hellerman & Stock [1938], using high concentrations of enzyme acting over long periods of time, obtained clearly positive results. Taken as a whole the work cited permits only one conclusion—that sufficiently active preparations of arginase do actually produce urea from argininic acid. With this conclusion our own experience is in complete conformity.

In the present paper the action of arginase upon argininic acid is examined further from the following points of view:

(1) In all previous work proof of hydrolysis has rested solely upon the production of urea. Although the cogency of such evidence cannot reasonably be doubted, it seemed worth while to demonstrate the appearance of the other obligatory product of arginase action, α-hydroxy-δ-amino-*n*-valeric acid. This was the more desirable, because Hellerman & Stock [1938] have expressed doubt concerning the constitution of the substrate itself.

(2) According to Felix & Schneider [1938] the optimum *p*H for the action of arginase on argininic acid is 6·8–7·0. So great a divergence from the optimum for arginine itself (*p*H 9·8) would, if confirmed, have considerable theoretical interest.

(3) The precise quantitative effect upon arginase efficiency of various substitutions in the substrate molecule is known for only one or two examples. To these we have now attempted to add the case of argininic acid.

Experimental

Preparation of the substrate. Argininic acid in 58% yield was prepared from *l*(+)-arginine hydrochloride [Felix & Schneider, 1938]. The product, which gave a negative ninhydrin test, melted at 225° (Felix & Schneider give 228°). Found: N, 23·8% (Kjeldahl); $C_6H_{13}O_3N_3$ requires 24·0%. $[\alpha]_D^{25°} = -11·85°$; $c = 10·0$; $l = 2·2$; $\alpha = -2·61°$; Felix & Müller [1928] found $[\alpha]_D^{20°} = -12·5°$ (no details).

Preparation of the enzyme. A stable, arginase-rich and relatively pure dry powder was prepared by heating a 1 : 1 aqueous extract of liver to 58°, filtering from coagulated protein, and precipitating with 1 to 1½ volumes of acetone. The precipitate was separated by centrifuging, and extracted repeatedly with water. The combined extracts were clarified and again precipitated with acetone. This procedure was repeated four or five times. The final acetone precipitate was then dried *in vacuo* and ground to a fine powder. In some preparations each intermediate acetone precipitate was dried before being re-extracted. This gave a product somewhat richer in arginase, but involved a greater over-all loss of the enzyme.

Active arginase solutions were obtained from the dry powder by shaking a portion for 30–45 min. with two or more times its weight of water. The extract was clarified by centrifuging, and, if a maximal recovery of enzyme was desired, the residue was extracted twice more. The several extracts were united, and diluted to any convenient volume. The quantities of water used at each extraction and the ratio of the final volume to the weight of powder extracted would depend upon the activity of the powder and the desired concentration of enzyme. With a triple extraction the highest conveniently attainable concentration was that in which each ml. of the final dilution contained the enzyme extractable from 0·2 g. of powder.

Examples for one particular specimen of arginase powder are shown in Table 1. All activities there recorded are those obtaining in the presence of an optimum concentration of Co [Hellerman & Perkins, 1935]. They were determined by the method of Hunter & Dauphinee [1930] and are expressed in their units. The data indicate that whether the volume of water used for extraction is large or small—whether, accordingly, the arginase concentration of the extract is low or high—the yield of enzyme per mg. of powder is much the same. Only two of the calculated yields in the last column differ significantly from 4·4 units per mg. The first aberrant figure is probably to be attributed to accident. The second suggests that when the volume/weight ratio is very low, extraction is not quite complete. The ready and uniform solubility of the enzyme is none the less sufficiently evident.

Table 1

mg. powder extracted per ml. extract	Dilution at which activity of extract was determined	mg. urea-N produced in 30 min. at 37°	Units of arginase	
			per ml. undiluted extract	per mg. powder
1·6	Undiluted	1·97	6·7	4·2
10	1 in 5	2·12	37·8	3·8
26·4	1 in 15	2·18	119	4·5
49	1 in 25	2·36	226	4·6
102	1 in 50	2·31	435	4·3
204	1 in 125	1·80	725	3·6

The activity of the preparation of Table 1 is only moderate and we have prepared others having extractable activities up to 8·5 units per mg. From these it was easy to obtain clear arginase solutions with activities of > 1000 units per ml.

Sometimes it is convenient to use the powder directly without extraction. On such occasions we have assumed the activity of the whole powder to be the same as that of its triple extract: a not wholly justifiable assumption, for a suspension of the powder is usually somewhat more active than a centrifuged extract.

*Isolation and identification of α-hydroxy-δ-amino-*n*-valeric acid.* The method already used in the analogous case of carbamido-arginine [Hunter, 1938] was most suitable. Urea was destroyed by urease as it was formed, and after an adequate period of digestion the mixture, freed from inorganic ions, was concentrated, until organic solutes crystallized. The material thus obtained was then recrystallized until free from any unaltered substrate.

In one successful experiment 10·8 g. argininic acid, 1·6 g. arginase powder (5 units per mg.), 15 ml. of a highly active urease solution, and 0·5 mg. $CoCl_2$, $6H_2O$, were dissolved in 250 ml. water. The mixture was made just alkaline to phenolphthalein with NH_3, treated with toluene and incubated at 37° for 72 hr. It was then found to contain a total of only 0·326 g. of NH_3-N. Since part of this had been added as such, it was evident that decomposition of argininic acid was proceeding very slowly. Accordingly 4·8 g. more of arginase powder were added, making a total of 6·4 g. or 32,000 units of the enzyme. After a further incubation period of 120 hr. NH_3-N had risen to 1·046 g., a gain of 0·72 g. Since the complete hydrolysis of the substrate would have yielded urea containing 1·725 g. of N, it appeared that the degree of hydrolysis now attained was >42 %, but <60 %. For 50 %, the quantity of α-hydroxy-δ-amino-valeric acid to be expected was 4·1 g.

On removal from the incubator the mixture was made just acid to Congo red with H_2SO_4, boiled to coagulate proteins, treated with an excess of baryta water, filtered, freed from NH_3 by vacuum distillation and then from Ba^{++} with the exact amount of H_2SO_4. The filtrate from the $BaSO_4$, after concentration *in vacuo*, was decolorized with norite, further concentrated to a volume of about 8 ml., and treated with enough alcohol (23 ml.) to produce a slight turbidity. Crystallization, which soon became evident, was promoted by adding more alcohol and cooling to −10°: first crop 0·59 g. On evaporating the mother liquor to dryness, taking up in a small quantity of water, and adding alcohol to a concentration of about 70 %, the second crop (0·6 g.) separated; it consisted of large glittering cubes, which gave a completely negative response to the Sakaguchi test for the guanidine group. The first crop gave a positive reaction, and was therefore presumably contaminated with unaltered argininic acid; but after a single recrystallization the Sakaguchi reaction disappeared, and the material presented the same rather characteristic appearance as the second crop. The total yield of pure product was 0·86 g., 21 % of that calculated.

The substance thus isolated was freely soluble in water, hardly at all in ethanol. Its aqueous solution was acid to litmus. From water or from 70 % ethanol it crystallized in remarkably hard, clear, shining parallelepipeds with rectangular (often square) outlines, reaching sometimes a length of 2–3 mm. Its composition agreed with that of the expected α-hydroxy-δ-amino-valeric acid. (Found: C, 45·6; H, 8·25; N (Dumas), 10·7; amino-N (Van Slyke), 10·6 %. $C_5H_{11}O_3N$ requires: C, 45·1; H, 8·33; N, 10·53; amino-N, 10·53 %.) It melted (after another recrystallization) at 196·4° (corr.), and the aqueous solution was laevorotatory ($[\alpha]_D^{21°} = -17·1°$; $c = 10·02$; $l = 1$ dm., $\alpha = -1·71°$).

According to Fischer & Zemplen [1909] α-hydroxy-δ-amino-valeric acid, heated for a short time at its M.P., is converted into an anhydride, 3-hydroxy-

2-piperidone, which is readily soluble in hot ethyl acetate. When treated in this way, our enzymic product was observed to yield a sublimate, which condensed in needles several mm. long. A hot ethyl acetate extract of the unsublimed residue deposited upon cooling a further crop of crystals identical with the sublimate. This material was an anhydride. (Found: C, 52·16; H, 7·85; N, 12·11%. $C_5H_9O_2N$ requires: C, 52·16; H, 7·88; N, 12·16%.) It was extremely soluble in water and in ethanol, freely soluble in hot, but sparingly in cold, ethyl acetate. From water it crystallized sometimes in the form of hexagonal plates, but usually as long thick prisms with obliquely pointed ends. From alcohol or ethyl acetate it separated as a close meshwork of long and delicate needles, M.P. 171·5° (corr.). The aqueous solution was neutral and laevorotatory ($[\alpha]_D^{21°} = -6·0°$; $c = 7·66$; $l = 1$ dm., $\alpha = -0·46°$).

An amino-acid of formula $C_5H_{11}O_3N$ and M.P. 193–194° has been described by Felix & Müller [1928] as the product of the decomposition of *l*-argininic acid by $Ba(OH)_2$. This acid, when melted, yielded, partly as a sublimate, an anhydride, M.P. 169°. There can be no doubt that these substances, taken to be respectively α-hydroxy-δ-amino-valeric acid and 3-hydroxy-2-piperidone, are the same as those now obtained through the use of arginase. Felix & Müller make no mention of optical activities, but otherwise their description, including M.P., tallies exactly with our own observations.

On the other hand, our products differ in certain respects from the synthetic (optically inactive) α-hydroxy-δ-amino-valeric acid and 3-hydroxy-2-piperidone of Fischer & Zemplen [1909], with original samples of which we were able directly to compare them. Before the comparison was made, the samples were repeatedly recrystallized, the acid from water and alcohol, the anhydride from ethyl acetate, to constant M.P. The acid then resembled our own in its solubilities, in its conversion upon melting into an anhydride and in the fact (not recorded by Fischer & Zemplen) that the anhydride readily sublimed. On the other hand, the M.P. was 188·2° (according to Fischer & Zemplen, 188–191°) not 196·4°, and it crystallized in a variety of shapes—thick hexagonal or rhomboid plates, long straight-ended hexagonal prisms, oblong plates with bevelled edges and corners—but never in rectangular parallelepipeds. As for the anhydride, it had the same general properties as ours, and, like it, crystallized from ethyl acetate in a characteristically dense meshwork of long fine needles; but it melted at 134·3° (Fischer & Zemplen say 141–142°) instead of at 171·5°. The observed differences, whether of M.P. or of crystalline form, are just such as might exist between an optically active substance and its racemate. The evidence suggests therefore that Fischer & Zemplen's substances are racemic compounds, of which ours (as well as Felix & Müller's) are the respective laevorotatory components.

Prof. M. A. Peacock of the Department of Mineralogy, University of Toronto, has been kind enough to compare for us some of the crystallographic properties of the two acids. In his report he says: 'The optical properties and the X-ray powder photographs of the tabular crystals prepared by Hunter & Woodward and the prismatic crystals of Fischer & Zemplen are similar, but not identical. In view of the dissimilarity of the crystal structures of tartaric and racemic acids [Astbury, 1923, 1, 2], the differences noted in the present compounds do not conflict with the conclusion that they are the active and the racemic forms respectively.'

The evidence therefore confirms the constitution hitherto assigned to argininic acid. No doubt then remains that arginase can attack, however feebly, a substance in which the α-amino group is lacking.

Effect of p*H on the action of arginase upon argininic acid.* In numerous experiments we determined the effect of varying *p*H upon the amount of argininic acid decomposed within a given time by a given quantity of arginase. In some the enzyme was activated by additions of Co, in others it was not. Since all gave concordant results, it is needless to report more than two. In each of these a series of appropriate *M*/4 phosphate-phenolsulphonate buffers [Hunter & Morrell, 1933], in 2 ml. portions, were mixed with 2 ml. of a 3·125 % solution of argininic acid and 2 ml. of a fresh extract of arginase powder. The added extract contained, in Exp. A, 400 units of arginase together with 0·35 mg. $CoCl_2$, $6H_2O$; in Exp. B, $3\frac{1}{2}$ times as much enzyme, but no Co. All mixtures were made up in duplicate. One member of each pair was used for the determination of *p*H by the glass electrode (Beckman). The others were incubated at 37° for 24 hr., the urea produced being determined by the method of Van Slyke & Cullen [1914]. It will be evident that each mixture contained, in a total volume of 6 ml., 62·5 mg. of argininic acid. The maximum amount of urea-N to be expected was therefore 10 mg.

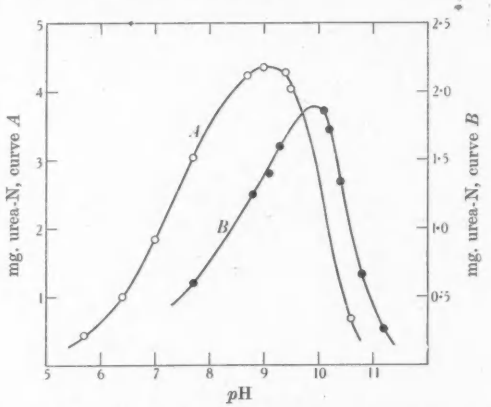

Fig. 1. Relation between *p*H and the action of arginase upon argininic acid. Curve *A*, with its urea scale on the left, shows this relation in the presence of Co; curve *B* (urea scale on the right) in its absence.

The results are shown graphically in Fig. 1. The curves merely represent enzyme actions for equal times and are not strictly *p*H-activity curves, but they suffice to show the general nature of the *p*H effect. Both curves, it is evident, are highly unsymmetrical, with descending limbs much steeper than the ascending. For the unactivated enzyme (curve *B*) the peak of action is at or near *p*H 9·9, the very point at which arginase shows its maximal activity towards arginine [Hunter & Morrell, 1933]. In the presence of Co the whole curve (*A*) is shifted to the left, so that the optimum *p*H is now about 9·1. A similar, if rather more pronounced, effect of Co has been observed in the action of the enzyme upon arginine [Hellerman & Stock, 1938; Damodaran & Narayanan, 1940]. Whether then the substrate be arginine or argininic acid, the relation between *p*H and arginase action is shown to be essentially the same.

Relative activities of arginase towards arginine and argininic acid. It is evident, from the experiments already reported, that in any liver extract or arginase concentrate the ratio (activity towards argininic acid)/(activity towards arginine)

must be very small. In an endeavour to estimate it, we have applied the principle already used [Hunter, 1938] with the substrate carbamido-arginine. This involves a determination and comparison of the quantities of enzyme required to perform, in some chosen time, identical amounts of work upon equivalent quantities of the respective substrates. On the assumption that the velocity curve of the reaction has the same form with each of the substrates, the ratio of the enzyme quantities found will be the reciprocal of the activity ratio sought.

In the present instance this principle was utilized in the following manner. An arginase solution was prepared containing (after activation by Co) about 750 units of enzyme per ml. 1 ml. of this solution was pipetted (*a*) into a urea tube, (*b*) into an ordinary test-tube. To each 1 ml. portion were added 7·5 mg. $Co(NO_3)_2, 6H_2O$ in 0·15 ml. water (a 5% solution). This addition was in the nearly optimum proportion of 1 mg. per 100 units of enzyme. Since, according to unpublished observations, the activation is progressive, the mixtures were left at room temperature overnight to 'ripen'.

Next morning there were added to (*a*) 3 ml. of a 2·084% solution of argininic acid and 2 ml. of a 0·25 *M* phosphate-phenolsulphonate buffer of *p*H 8·4 [Hunter & Morrell, 1933]. The mixture was covered with toluene, and placed in a thermostat at 37° for 24 hr. Meanwhile the other enzyme mixture, (*b*), was diluted to 1000 ml., and portions of 0·2, 0·4, 0·6, 0·8 and 1·0 ml. were measured into urea tubes, each of which already contained 3 ml. of a 2·506% solution of arginine hydrochloride, 2 ml. of the above-mentioned buffer solution, and enough water to make (after the addition of the enzyme solution) a total volume of 6 ml. To each of these mixtures there was further added, in order to ensure the maintenance of maximal activity, a drop of 2% $Co(NO_3)_2$. The arginine mixtures were then treated exactly like the argininic acid one. At the end of the 24 hr. incubation period the urea contents of all six tubes were determined in the usual way.

A parallel experiment was also made, in all respects identical except that the preliminary treatment of the enzyme with Co, as well as the addition of Co to the incubating mixtures, was omitted. Each of the 12 mixtures incubated contained in 6 ml. (or slightly more) the same quantity of buffer salts and arginine or argininic acid equivalent to 10 mg. of urea-N.

The results of two such experiments are recorded in Table 2, all enzyme quantities being there expressed in terms of the original undiluted arginase solution.

Table 2

		mg. urea-N			
		Without Co		With Co	
Substrate	ml. undiluted arginase solution	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Arginine	0·0002	0·54	0·54	2·10	2·06
	0·0004	0·99	1·01	3·38	3·37
	0·0006	1·41	1·44	4·47	4·44
	0·0008	1·69	1·76	5·16	5·11
	0·001	2·00	2·06	5·89	5·88
Argininic acid	1·0	0·76	0·69	2·49	2·58

Inspection shows that in each of the four instances the work done upon argininic acid by 1 ml. of enzyme would have been accomplished upon an equivalent amount of arginine by 0·0002–0·0004 ml. Interpolating graphically for each in turn of the four groups of data available, we obtained the results in the second line of figures in Table 3. Since the volume of arginase solution used

with argininic acid was always 1 ml., these results are numerically the same as the activity ratios; but it is more convenient to express these in the form shown in the last line of the table, with the activity towards argininic acid taken as unity.

Table 3

	Unactivated		Activated	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
mg. urea-N produced from argininic acid by 1 ml. arginase	0·76	0·69	2·49	2·58
ml. arginase to produce same amount of urea-N from arginine	0·00029	0·00026	0·00025	0·00027
Activity ratio (argininic acid : arginine)	1 : 3450	1 : 3850	1 : 4000	1 : 3700

The four values found for the activity ratio are as consistent as could reasonably be expected; and it seems safe to conclude that, under the conditions given, arginase is from 3500 to 4000 times less active in the hydrolysis of argininic acid than in that of arginine. None of the estimates differs more than 8% from the average of 1 : 3750.

The precise value of the activity ratio is less important than the demonstration, here afforded, that it is not altered by Co activation of the arginase. With Co the average ratio is 1 : 3850, without it 1 : 3650. As has been argued already for carbamido-arginine [Hunter, 1938] and canavanine [Kitagawa & Eguchi, 1938; Damodaran & Narayanan, 1940], constancy of the ratio under varying conditions is strong evidence for the identity with arginase of the enzyme attacking the alternative substrate. In the present experiment the degree of activation attained was very high. From the data for Exp. 2 in Table 2 it may be seen that exactly the same amount of urea was produced from arginine by 0·0002 ml. of activated enzyme as by 0·001 ml. of the unactivated. The specific arginase activity was increased, therefore, no less than five times (and possibly slightly more in Exp. 1). That the activity ratio nevertheless remained unaltered, means that the agent attacking argininic acid was activated in precisely the same rather remarkable degree. It seems unlikely then, to say the least, that it could have been a separate entity.

(We have lately found reason to suspect, that under the prevailing conditions the effect of Co was less one of direct activation, than of protection against spontaneous inactivation. Should this prove to have been the case, the phraseology of the preceding paragraph would have to be modified, but the general argument would not be affected.)

Discussion

Since liver extracts have now been shown to produce from argininic acid not only urea, but also a substance identifiable, with reasonable certainty, as *l*-α-hydroxy-δ-amino-*n*-valeric acid, there can remain little doubt (1) that argininic acid is actually α-hydroxy-δ-guanido-*n*-valeric acid, or (2) that it is hydrolysable by a liver enzyme having an action similar to that of arginase. The identification of this enzyme with arginase itself is justified by the facts (1) that whether the substrate be argininic acid or arginine the relation between *p*H and enzyme activity is of the same unsymmetrical form, (2) that for each substrate the optimum *p*H lies close to 9·8, (3) that with both, activation by Co shifts the optimum to a less alkaline region, and (4) that activation enhances the rate of hydrolysis in each case to an equal degree. The demonstration would

seem to be complete, that replacement of the α-amino group of arginine by hydroxyl leaves a substrate still accessible to attack by arginase.

In Fig. 1 the highest degree of hydrolysis shown is 44%; but under more extreme conditions we have found it possible to reach a figure of 91%. This is not very different from the maximum of 85·6% reported by Calvery & Block [1934].

As a striking example of the bearing of chemical structure upon the concept of specificity, the action of arginase upon argininic acid has considerable theoretical interest. Physiologically it can have little if any significance. It is known already that a single substituent in the amino group of arginine notably reduces its 'affinity' for the enzyme. In one instance in which this effect was studied quantitatively—that of carbamido-arginine—the activity of the enzyme towards the substituted compound was 165 times less than its activity towards arginine [Hunter, 1938]. The complete replacement of the amino group has, it would now appear, a depressant effect more than 20 times greater. An activity-ratio of 1 : 3750 implies—to put it in terms of time—that a given amount of arginase would take 3750 hr. or almost exactly 5 months, to liberate from argininic acid as much urea as it would produce in a single hour from an equivalent quantity of arginine. The calculation gives quantitative emphasis to the conclusion of Hellerman & Stock [1938]—that the α-amino group, though not wholly indispensable, is necessary for the *efficient* functioning of the enzyme.

Arginase, it is known, exerts a feeble action not only upon *l*-argininic acid, but also upon *dl*-δ-guanidino-valeric acid, which has no substituent at all in the α position [Hellerman & Stock, 1938; Richards & Hellerman, 1940], and upon *d*(−)-arginine, in which the amino group is in the 'unnatural' configuration [Edlbacher & Zeller, 1936]. No determination of activity-ratios for these substrates have yet been made; but published data, as far as they go, suggest that they will be found to be of the same order as that for argininic acid. Should this prove to be the case, one might reasonably deduce that neither an α-hydroxy nor a sterically misplaced α-amino group offers any 'point of attachment' whatever for arginase; and that the effect of that enzyme upon *d*(−)-arginine is not so much an instance of imperfect optical specificity as another proof that the amino group as such, apart altogether from its spatial position, is not an essential feature of the substrate's structure.

Some comment appears to be called for upon the discrepancy between the optimum *p*H as here reported and that given by Felix & Schneider [1938]. This discrepancy would seem to be another example of the fact that the apparent optimum is apt to be considerably affected by the conditions under which it is determined [Hunter & Morrell, 1933]. In any case we have been informed, in a personal communication from Prof. Felix himself (January, 1939), that a repetition of his experiments in another laboratory indicated, in sufficient agreement with our own observations, an optimum at *p*H 9·4. It would not be surprising if the optimum for arginase should sometimes be found to be dependent upon the nature of its substrate; but the substitution of —OH for $—NH_2$ in the alpha position is shown to be in this respect without appreciable influence.

SUMMARY

Argininic acid is split by liver extracts into urea and *l*(−)-α-hydroxy-δ-amino-*n*-valeric acid. The latter product has been isolated and identified. Under favourable conditions 90% of the substrate may be hydrolysed. The enzyme responsible is identical with arginase, because (1) it has the same *p*H optimum and (2) in the presence of Co its action is magnified in exactly the same degree.

Susceptibility to arginase is therefore not absolutely dependent upon the presence of an intact or a modified α-amino group. This group exerts, nevertheless, an extremely important influence; for argininic acid is hydrolysed by arginase about 3750 times less actively than arginine.

We are indebted to Dr Max Bergmann of the Rockefeller Institute for Medical Research, New York City, for permitting Mr Stephen M. Nagy to analyse for us a sample of our 3-hydroxy-2-piperidone. The analysis of the parent acid was made by Mr Edson of the Department of Biochemistry of this University. The specimens of these substances prepared by Fischer & Zemplen we owe to Dr H. O. L. Fischer of the Department of Chemistry. We have to thank Dr M. A. Peacock of the Department of Mineralogy for his crystallographic report. In the determination of the activity ratio we had the technical assistance of Mr Clarence E. Downs. To all of these helpers we now make grateful acknowledgement.

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146. DERIVATIVES OF SULPHANILAMIDE AND CHOLIC ACID

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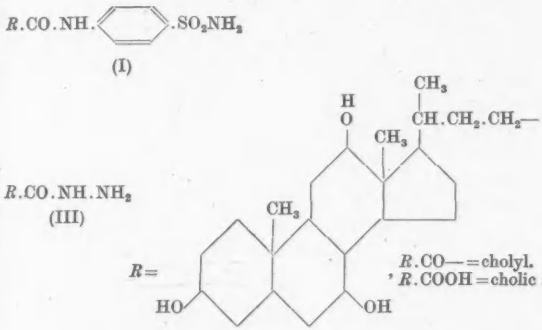
It was suggested to the author by Prof. J. H. Dible that use might be made of sulphanilamide derivatives which would be concentrated in the bile. It seemed possible that compounds between cholic acid and sulphanilamide might be so excreted, and the following substances have therefore been prepared: N-(p-*sulphonamido*) *phenyl cholamide* (I), and (probably) *α-cholyl β-p-aminobenzene-sulphonyl hydrazine* (II).



The compound (I) was isolated, apparently as a hydrate, after direct condensation of sulphanilamide with triformyl cholyl chloride, as prepared by Cortese & Baumann [1935].

For the preparation of the hydrazine derivative (II), theoretically equivalent amounts of pure cholyl hydrazine ('cholic acid hydrazide', III, Bondi & Müller [1906]; Caldwell [1938]) and pure *p*-acetamidobenzenesulphonyl chloride were mixed in pyridine solution. Reaction took place at once, but the product could not be isolated in crystalline form and free from solvent. Hydrolysis gave (II) as a white, possibly crystalline, powder which, by titration, diazo-colour measurement and elementary analysis appeared to be pure. It could not, however, be recrystallized from solvents and had no definite melting point; such behaviour is, of course, not uncommon among cholic acid derivatives. For further characterization, the compound (II) or the original condensation product was boiled with alkali. There was thus obtained an acid, decomposing at 160°, which was also the product of alkaline hydrolysis of cholyl hydrazine (III). At *ca.* 80°, this acid seemed to lose solvent and was then indistinguishable from cholic acid. On oxidation with chromium trioxide, it was readily changed into dehydrocholic acid, which was also isolated by direct oxidation of (II) or of the original condensation product.

Finally, for comparison with (II), there were synthesised *α-benzoyl β-p-acetamidobenzenesulphonyl hydrazine* (IV, R_1=CO.CH_3), and *α-benzoyl β-p-aminobenzenesulphonyl hydrazine* (IV, R_1=H). The method used was the same as for the preparation of (II), and both products were crystalline and easily purified. Comparison by titration of the amino-compound (IV, R_1=H) with (II) gave the expected result.

Condensation of *p*-acetamidobenzenesulphonyl chloride with the 'hydrazides' (such as III) appears to offer a new and simple method for the preparation of derivatives of sulphanilamide and non-ketonic carboxylic acids. The products here described seem to be stable; they are sufficiently acidic to dissolve in ammonia and sodium carbonate solutions.

Preliminary tests by Prof. Dible indicate that the substance (II) has considerable bacteriostatic activity *in vitro*.

Experimental

All melting points are uncorrected. Elementary analyses and Rast molecular weight determinations were by Dr G. Weiler.

N-(p-*Sulphonamido-*)*phenyl cholamide* (*I*). Triformyl cholic acid [cf. Cortese & Baumann, 1935] was prepared by warming 20 g. of cholic acid with 40 ml. of 98/100 % formic acid at 40° until solution occurred. After 16 hr. at room temperature, the mixture was diluted with water and stirred until the product solidified. The collected and washed solid was dissolved in alcohol, warmed to *ca.* 60° and diluted, with mixing, until crystals formed, when gradual cooling gave the formyl compound as needles of M.P. 204–206°. Yield, 12–14 g.

The above compound (2 g.), dissolved in redistilled thionyl chloride (2 ml.), was kept at 0° for 1 hr. Dilution with light petroleum gave a gum, which was washed and rubbed with fresh petroleum until it formed a white powder. This was dried in air and used at once. 0·5 g. of formyl derivative was thus converted into the chloride, and the product was heated with 0·5 g. of sulphanilamide in 3 ml. of pyridine for 1 hr. at 100°. The cooled mixture was diluted with HCl solution, and the precipitate was washed and then warmed for 15 min. with 25 ml. of 5*N* NaOH. After re-acidification with HCl, the gelatinous product was collected, washed and dissolved in alcohol. Considerable dilution with water gave small white needles (0·2 g.) of M.P. 238–245°. Recrystallization gave substance (I) of M.P. 244–246°, decomp. (Found: C, 61·8; H, 8·3; N, 5·0; S, 5·0 %. $C_{30}H_{46}O_6N_2S.H_2O$ requires C, 62·1; H, 8·3; N, 4·8; S, 5·5 %.)

Further heating of the pyridine mixture lowered the yield of final product, which, in any case, was not always obtained in the amount mentioned above. Compound (I) could be separated from more acidic material by saturation of an alkaline solution with CO_2, when (I) was precipitated. The compound gave a red colour in the Liebermann-Burchard test; it was easily soluble in alcohol (from which it only crystallized after great dilution of a cold solution with water), in acetone, acetic acid and solutions of KOH, NaOH etc. Alkaline solutions very readily formed gels on cooling, even at great dilution, e.g. 50 mg./100 ml.

*α-Cholyl β-*p*-aminobenzenesulphonyl hydrazine* (*II*). A suspension of cholic acid (20 g.) in ethyl alcohol (150 ml.) was saturated with diazomethane. After evaporation of solvent, the solid residue was heated under reflux for 20 hr. with 5 ml. of pure hydrazine hydrate and enough alcohol to dissolve the mixture. The solid contents of the flask were then washed out with hot alcohol, and after considerable dilution with water there slowly separated long needles (16 g.) of cholyl hydrazine (III), of M.P. 193–195° [compare Bondi & Müller, 1906]. An identical product was obtained by the same procedure from triformyl cholic acid.

Cholyl hydrazine (10 g.) in pyridine (50 ml.) was mixed with a solution of *p*-acetamidobenzenesulphonyl chloride (5·5 g., M.P. 150–151°) in pyridine (50 ml.). The temperature rose at once to 40°. After 15 min. the solution was poured, with stirring, into excess of dilute HCl. A slightly yellow solid separated in almost quantitative yield. After collection, washing and drying at room temperature *in vacuo*, and at 50–60°, this product was purified by solution in acetone, filtration from a small amount of yellow insoluble matter and precipitation with light petroleum. The solid which formed appeared crystalline; it softened at *ca.* 160° and decomposed with frothing above 180°.

This substance gave a red colour in the Liebermann-Burchard test and a negative diazo-test; it dissolved readily in alcohol, acetone, acetic acid and solutions of NaOH, KOH, NH_3 and Na_2CO_3, from which it was precipitated with CO_2.

The whole of the wet crude product from a preparation as above was dissolved in 2*N* NaOH (150 ml.) and refluxed for 45 min. The cooled solution was diluted to *ca.* 500 ml. and saturated with CO_2. The white solid precipitate was collected, washed and dried *in vacuo* and then at 75°. Yield, 7 g. of compound (II).

The bicarbonate mother liquors were acidified with HCl, and the precipitated acid was collected, washed and purified by solution in dilute $NaHCO_3$. Acidification of the filtered $NaHCO_3$ solution gave 1·5 g. (dried) of acid which, from dilute alcohol, formed long white needles that frothed at 160°. On drying at 70–80°, these appeared to lose solvent and then had M.P. 194–196°, not depressed by cholic acid (M.P. 196–197°). The same acid was isolated when compound (II) (0·2 g.) was boiled for 1·5 hr. with 2*N* NaOH (2 ml.), or when cholyl hydrazine (1 g.) was refluxed for 1 hr. with 2*N* NaOH (10 ml.). The acid was oxidized in the usual way to dehydrocholic acid, M.P. 232–234°, décomp. Compound (II) was purified by solution in acetone and precipitation with light petroleum, followed by solution in dilute NaOH and precipitation with CO_2. After drying, it melted at *ca.* 150° and decomposed with frothing above 200°. (Found, on a sample dried to constant weight at 80° *in vacuo*: C, 62·5; H, 8·4; N, 7·2; S, 5·4%; mol. wt. (by titration and comparison with (IV), R_1 = H) = 573,545. $C_{30}H_{47}O_6N_3S$ requires C, 62·4; H, 8·1; N, 7·3; S, 5·5%; mol. wt. = 577.)

The substance gave a red colour in the Liebermann-Burchard test; it dissolved readily in alcohol, acetone, acetic acid, and also in NaOH, KOH, Na_2CO_3 and NH_3 solutions, from which it could be precipitated with CO_2. It was almost insoluble in water, chloroform, benzene, ether and ethyl acetate, and in dilute acids.

A solution of (II) in 30% alcohol gave quantitatively the amount of colour, calculated on a molecular weight basis, as sulphanilamide similarly treated by the diazotization method of Bratton & Marshall [1939]. Results on both compounds by this method were not exactly reproducible.

For oxidation, a solution of 0·2 g. of (II) in 2 ml. of acetic acid was cooled in water and treated gradually with 1·5 ml. of a solution of CrO_3 (2 g. dissolved in the minimal amount of water and brought to 10 ml. with acetic acid). Reaction with effervescence and formation of a precipitate occurred immediately. A little water was added to dissolve the precipitate and the mixture was kept for 15 min. at room temperature. It was then poured into excess of water and left overnight. The precipitate was collected, washed and stirred with dilute $NaHCO_3$. The filtered solution was acidified with HCl, and the precipitate collected, washed and recrystallized from dilute alcohol, from which it formed long needles of M.P. 229–232°, decomp., not depressed by dehydrocholic acid. The same acid was similarly obtained from the original condensation product.

α-Benzoyl β-p-acetamidobenzenesulphonyl hydrazine (*IV*, $R_1 = CO.CH_3$). Benzoyl hydrazine (1 g.) and *p*-acetamidobenzenesulphonyl chloride (1·7 g.) were mixed in pyridine (10 ml.). The temperature rose to 47°. After 15 min., the mixture was diluted with HCl and water and the precipitated solid collected, washed and recrystallized from dilute alcohol, from which it formed white needles of M.P. 219–220°, decomp. Yield, 1·8 g. (Found: C, 53·9; H, 4·5; S, 10·0%; mol. wt. (Rast) 315. $C_{15}H_{15}O_4N_3S$ requires C, 54·1; H, 4·5; S, 9·6%; mol. wt. 333.)

α-Benzoyl β-p-aminobenzenesulphonyl hydrazine (*IV*, $R_1 = H$). The above substance (IV, $R_1 = CO.CH_3$) (1 g.) was refluxed with 2*N* NaOH (10 ml.) for 20 min. The mixture frothed, became cloudy and evolved an almond odour. After dilution, it was saturated with CO_2, when crystals were deposited. These were collected, washed and recrystallized from dilute alcohol, from which (IV, $R_1 = H$) separated as long white needles of M.P. 190–192°, decomp. Yield, 0·4 g. (Found: C, 54·2; H, 4·5; N, 14·4; S, 11·4%; mol. wt. (Rast) 316. $C_{13}H_{13}O_3N_3S$ requires C, 53·6; H, 4·5; N, 14·4; S, 11·0%; mol. wt. 291.)

After treatment with HCl, the bicarbonate liquors from the above hydrolysis yielded a small amount of benzoic acid. With a solution of 2:4-dinitrophenylhydrazine in HCl, they gave the 2:4-dinitrophenylhydrazone of benzaldehyde.

Compound IV ($R_1 = H$) gave a positive diazo-reaction; it dissolved in dilute acids and alkalis.

SUMMARY

For therapeutic trial, there have been prepared two compounds of cholic acid and sulphanilamide. One of these substances has a free $—SO_2.NH_2$ group, the other a free $—NH_2$ group. The latter derivative was made by reaction between cholic acid 'hydrazide' and *p*-acetamidobenzenesulphonyl chloride. It is suggested that this simple method can be used generally for preparation of compounds of sulphanilamide and non-ketonic carboxylic acids. For comparison, and as a further example, the corresponding benzoic acid derivative was synthesized.

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ERRATUM

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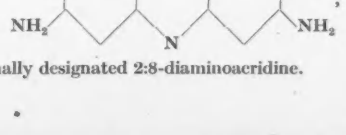
The compound referred to as 2:7-diaminoacridine is



normally designated 2:8-diaminoacridine.

147. A NUTRITIONAL INVESTIGATION OF THE ANTIBACTERIAL ACTION OF ACRIFLAVINE

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STUDIES of the mode of action of chemotherapeutic agents, based on the hypothesis that they interfere with metabolites essential to pathogenic organisms, have recently been successful in interpreting the antibacterial action of sulphanilamide [Woods, 1940] and mercuric salts [Fildes, 1940, 1], and in suggesting lines for the development of further therapeutic agents [Fildes, 1940, 2; McIlwain, 1940, 2, 3]. In these papers [see also McIlwain, 1941] it is shown that inhibitory agents cause fresh nutritional needs in the affected organisms, and that when these are satisfied growth can take place in the presence of an otherwise inhibitory concentration of therapeutic agent ('reversal' of the inhibitor). There is a general realization that therapeutic agents may act by affecting metabolic processes, and the present method of investigating their action is possible only because more exact knowledge of bacterial nutrition is now available. Thus Ehrlich [see Muir, 1915–16] suggested that the normal function of the 'receptors' was nutritive, though he later abandoned, or failed to emphasize, this view [Ehrlich, 1909; 1913]. Metabolic ideas have more recently been applied to acridine compounds by Quastel & Wheatley [1931] and by Dickens [1936]. Analogous conceptions of enzyme interference have also successfully explained the pharmacological actions of eserine as due to blocking of choline esterase.

The acriflavine drugs were developed by Ehrlich as trypanocides, but their main current use is as antiseptics. *Bact. coli* and *Streptococcus haemolyticus* were the organisms mainly used in the present work. Acriflavine (here used as a general term) is a mixture of 2:7-diamino-acridine, proflavine, and its 9-methochloride which is sometimes, as in this paper, referred to as euflavine. Slight differences in the actions of the two compounds have been described. The present work was mainly carried out with euflavine; the conclusions were found applicable to proflavine.

EXPERIMENTAL

Each test comprised a tube in which the organisms were grown from a small inoculum in a chemically defined liquid medium, a second in which growth was prevented for several days by a suitable concentration of acriflavine component, and further tubes which contained all the above materials together with different concentrations of additional nutrients whose relation (as 'reversing factors') to acriflavine inhibition was being studied.

Bact. coli will grow in a simple medium containing per tube of 10 ml.: KH_2PO_4, 45 mg.; $(NH_4)_2SO_4$, 5 mg.; NH_4Cl, 5 mg.; Na lactate, 28 mg.; Fe and Mg salts, traces; NaOH to *p*H 7·6. For specific reversal effects it was necessary to add further nutrients; in the present experiments these comprised: glucose, 12 mg.; aneurin, nicotinamide and β-alanine, 12μg.; pimelic acid, uracil, cytosine, thymine and guanine, 25μg.; methionine and tryptophan, 125μg.; cystine,

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600μg. The inoculum of about 1000 organisms, grown in the simple medium, was added last, and the tubes were incubated in a 5% CO_2-air mixture at 37°. *Streptococcus haemolyticus* (groups A and C) was grown according to McIlwain [1940, 1] in media containing thiolacetate, pantothenate concentrates (which contain other nutrients, probably biotin) and $2 \times 10^{-8} M$ riboflavin.

Differentiation of reversing effects

Various extracts of natural materials, of which some 30 were made, removed the inhibition of *Bact. coli* caused by addition of euflavine to the medium described above; such reversal was specific in the sense that the concentrations of extracts causing it were without visible effect on uninhibited growth (Table 1).

Table 1. *Differentiation of reversing factors*

Euflavine M ($\times 10^{-5}$)	Reversing factor: Material	Reversing factor: mg./ml.	Growth* after: Hours: 16	Hours: 24	Days: 2	Days: 3
0	0; or, (*a*) (see below)	150	+ + +	+ + + +	+ + + +	+ + + +
	(*b*) (see below)	4				
3 or 6	0	—	0	0	0	0
3	(*a*) Alkaline yeast† preparation (Type I)	150	+ + +	+ + + +	+ + + +	+ + + +
3	,,	30	+	+ + +	+ + +	+ + + +
3	,,	6	0	0	+ + +	+ + + +
6	,,	150	+ + +	+ + + +	+ + + +	+ + + +
6	,,	30	0	+	+ + +	+ + + +
6	,,	6	0	0	0	+ + +
3	(*b*) Tryptic casein (Type II)	4 or 0·8	+ +	+ + +	+ + +	+ + +
3	,,	0·16	+	+ + +	+ + +	+ + +
3	,,	0·032	0	+ +	+ + +	+ + +
6	,,	4	0	+	+ +	+ +
6	,,	0·8 or less	0	0	0	0

* Here and in other tables: organism, *Bact. coli*. Plus signs are proportional to mass of growth, estimated visually. Concentrations are those in final media.

† An alcohol precipitate of an alkaline extract of yeast; the figures refer to the weights of yeast from which the addenda were prepared.

The reversing activity was, however, complex in that it appeared due to at least two types of material differing in occurrence and in chemical and biological properties. Thus marmite or casein hydrolysates were very active (little material being necessary for reversal) against $3 \times 10^{-5} M$ euflavine, while an alkaline extract of yeast was much less active; against $6 \times 10^{-5} M$ euflavine, however, the alkaline extract of yeast was almost equally active, while more than 100 times the previous quantity of tryptic casein was not. The interrelations between inhibitor and reversing agents thus approximate to those named Types I and II in a previous nutritional study of inhibitory action [McIlwain, 1940, 2]. Analogy with the above types is not complete, as the reversing factors in the former investigation were also essential for uninhibited growth, but the points of similarity are: in the case of Type I action, the effects of increased inhibitor concentration can be reversed by a comparable increase in reversing agent, while in the case of Type II action such increase does not lead to comparable reversal.

Nucleic acid and nucleotides as Type I factors

The sources and modes of extraction which yielded most material reversing according to Type I, and the preliminary chemical reactions of the material, led to attempts to remove the inhibition of euflavine with nucleic acid. This was immediately successful. Purification of commercial yeast nucleic acid according to Gulland & Jackson [1938] and by Na acetate-alcohol precipitation, did not afford fractions of marked difference in activity. Thymus nucleic acid was equally active. Processes which degrade nucleic acid greatly reduced its activity (Table 2). It was concluded that the intact nucleic acid molecule is most effective in reversal, though this necessarily lacks the final confirmation of synthetic material. A lesser activity on the part of smaller nucleotides was confirmed by experiments with crystalline specimens of adenylic and guanylic acids, and with cozymase (Table 2).

Table 2. *Nucleotides as Type I reversing factors*

Substance added	Approx. molarity	Fluorescence before inoculation mg./ml.		Growth after (days) 1	2	3	5
0	—	—	+ + +	0	0	0	0
Na nucleate*	4×10^{-4}	0·5	0	+ + + +	+ + + +	+ + + +	+ + + +
	8×10^{-5}	0·1	+	+	+ + + +	+ + + +	+ + + +
	$1{\cdot}6 \times 10^{-5}$	0·02	+ +	0	+ +	+ + + +	+ + + +
Nucleic acid NH_4OH hydrolysate†	—	0·5	+	0	+ +	+ + + +	+ + + +
	—	0·1	+ +	0	0	+ +	+ + + +
	—	0·02	+ + +	0	0	0	0
Nucleic acid H_2SO_4 hydrolysate‡	—	0·5	+ + +	0	+ +	+ + +	+ + +
	—	0·1 or 0·02	+ + +	0	0	0	0
Adenylic or guanylic acids	$1{\cdot}5 \times 10^{-3}$	0·5	+ +	0	+ +	+ + +	+ + +
	3×10^{-4}	0·1	+ + +	0	0	+ + +	+ + +
Cozymase§	10^{-5}	—	+ +	0	+ + +	+ + + +	+ + + +
	2×10^{-6}	—	+ + +	0	0	+ +	+ +
Uninhibited control				+ + + +			

Inhibiting concentration of euflavine, $4 \times 10^{-5} M$.
* Commercial or purified yeast sodium nucleate, or thymus nucleate.
† By 2·5% NH_4OH autoclaved in aqueous solution at 10 lb./sq. in. for $\frac{3}{4}$ hr.
‡ By 2% aqueous H_2SO_4 refluxed for 2 hr.; concentrations given refer to the initial nucleate.
§ Not pure material.

Reversal of euflavine inhibition with purified nucleic acid took place also with *Bact. coli* in ordinary broth and in the simple ammonium lactate medium, and with *Streptococcus haemolyticus*. Inhibition due to proflavine was also removed.

Relation between Type I activity and euflavine fluorescence

The familiar greenish fluorescence of acriflavine solutions in visible light is shown well at the concentrations used in these tests. It was very noticeable in the present colourless media that this fluorescence sometimes disappeared with growth. The disappearance of fluorescence was not caused by removal of euflavine from the solution, which remained yellow, though such removal was caused to some extent by the bacterial cells. It could be simulated by the addition of acid, though the *p*H necessary was much lower than that attained during growth.

In mixing test solutions prior to inoculation it was observed that Type I sources, and nucleates, removed or reduced acriflavine fluorescence and that there was a direct correlation between such removal and the rapidity of subsequent growth (Table 2). As removal of fluorescence in acid solution is attributed to salt formation, it was considered that acriflavine probably formed with nucleic acids complex salts which, unlike those with simple acids, were stable at neutral *p*H. Mixing solutions of proflavine and nucleate showed this to be the case. In strong solutions a brown precipitate formed, with maximal precipitation from 4 mols. of proflavine to one of nucleate. To proflavine (0·5 g.) in water (100 ml.) was added yeast sodium nucleate (0·65 g.) in water (20 ml.). The precipitate was filtered after ½ hr., washed with water, dried *in vacuo*, ground to a red powder and further dried at 60° *in vacuo*; the product contained P, 5·98 % (calc. for $C_{38}H_{43}O_{28}N_{15}P_4 . 4C_{13}H_{11}N_3$, P, 5·85 %). In common with other nucleates, it was not crystalline, but a crystalline salt was prepared from adenylic acid. Proflavine base (0·1 g.) was added to a hot suspension of yeast adenylic acid (0·13 g.; found, P, 8·89 %; $C_{10}H_{14}O_7N_5P$ requires P, 8·94 %) in water (4 ml.) and the resulting brown solution filtered. Rosettes of small brown crystals which formed on cooling were filtered, washed with water and dried *in vacuo* at 60°: M.P. 189–191°; P, 5·54 % (calc. for $C_{10}H_{14}O_7N_5P . C_{13}H_{11}N_3$, P, 5·58 %). Qualitative evidence of complex formation was also obtained with thymus nucleic acid and guanylic acid.

Removal of fluorescence of dilute acriflavine solutions by nucleate does not occur with equivalent concentrations, but requires an excess of nucleate, as does also the reversal of acriflavine inhibition. Thus with $2·5 \times 10^{-4} M$ proflavine, 1·2 equiv., with $10^{-4} M$, 1·6 equiv., and with $10^{-5} M$, 3·2 equiv. of yeast nucleate were required for removal of visible fluorescence. This is attributed to dissociation of the salt. Salts of acridine itself with adenosine phosphoric acids are already known [Wagner-Jauregg, 1936].

Amino-acids as Type II factors

As fractionation proceeded it was found that Type II factor, unlike Type I, did not remove the fluorescence of acriflavine solutions. Marmite was the most readily available good source; the following were active, in decreasing order: casein hydrolysates; liver, heart and testicle extracts, especially press juice or cytolysates; potato, blood, serum and milk.

Marmite was fractionated by the techniques familiar in nutritional work, purification being followed by comparing the dry weight or amino-N (see (*d*) below) of fractions corresponding to 1 g. of marmite, with their reversing activity (see Table 3). The following successive processes yielded material of the activity described:

(1) *Starting material*: dry wt., 800 mg.; amino-N, 42 mg.

(2) *Lead acetate precipitation* at *p*H 3. Filtrate: amino-N, 36 mg.

(3) *Mercuric acetate precipitation* at *p*H 7. Decomposed precipitate: dry wt., 290 mg.; amino-N, 18 mg.

(4) *Butyl alcohol extraction* of dried material from Hg precipitate: amino-N, 7 mg.

(5) *Adsorption to alumina* from butyl alcohol-ether solution and elution with water. Eluate: dry wt., 27 mg.; amino-N, 3·6 mg.

The active material had the following properties.

(*a*) *Stability*. At stage 5, but not earlier, it was stable to autoclaving (120°, 20 min.). It was throughout stable to boiling with *N* or 20 % H_2SO_4 in an inert atmosphere, but not in air; thus though many protein hydrolysates were active

Table 3. *Concentrates and amino-acids as Type II factors*

Substance added	NH_2-N molarity	mg./ml.	Growth after: Hours 16–18	Hours 24	Days 2	Days 3
0	—	—	0	0	0	0
Marmite	3×10^{-3}	1	+ + +	+ + +	+ + +	+ + +
	6×10^{-4}	0·2	+	+ +	+ + +	+ + +
	$1{\cdot}2 \times 10^{-4}$	0·04	0	+ +	+ +	+ + +
Al_2O_3 eluate (stage 5)	$2{\cdot}6 \times 10^{-4}$	0·027	+ +	+ +	+ + +	+ + +
	$5{\cdot}2 \times 10^{-5}$	0·0054	+	+ +	+ + +	+ + +
	10^{-5}	0·0011	0	0	+ +	+ + +
Amino-acid mixture (see text)	10^{-3}	1·3	0	+	+ + +	+ + +
Phenylalanine	5×10^{-3}	0·84	0	0	+ + +	+ + +
	10^{-3}	0·168	0	0	+	+ + +
	Uninhibited control		+ + +	+ + + +	+ + + +	+ + + +

Inhibitory concentration of euflavine, 3×10^{-5} *M*.

when produced in N_2, they were little so when normally produced in air. Boiling with 10*N* NaOH in N_2 reduced the activity to $\frac{1}{2}$. It was not affected by catalytic reduction in acid solutions, by $NaHSO_2$ in acids or alkalis, nor by HIO_4.

(*b*) *Solubility*. The material could not easily be removed from aqueous solution by amyl or butyl alcohols, but these solvents, or ethyl alcohol, extracted it from dried material; ether or chloroform did not. The extractions were not markedly affected by *p*H between 3 and 10.

(*c*) *Adsorption* did not occur from aqueous solutions of *p*H 7, 10 or 3 (when this could be used) by norite, alumina, or fuller's earth when shaken with the adsorbent, but adsorption took place to columns of permutite from aqueous solution and to alumina from butyl alcohol.

(*d*) *Nitrous acid* destroyed activity in 2 hr. at room temperature but not in $\frac{1}{2}$ hr. at 0°. For this reason, amino-N determinations were used in following the concentration of the factor.

(*e*) *Acetylation* with acetic anhydride in slightly alkaline solution removed almost all activity; this was not restored by standing in dilute alkali but was restored by acid hydrolysis.

(*f*) *Esterification*. Alcoholic HCl produced at stage 5 an ether-soluble substance with delayed activity which became fully active on hydrolysis. On distillation of the ether-soluble substance no activity was found in fractions of B.P. (bath temperatures) $<100°/10$ mm. and very little $>100°/0{\cdot}002$ mm.; the most active fraction was that of B.P. 95–100°/0·008 mm.

(*g*) *Salts*. Phosphotungstic acid partly precipitated the material, but flavianic and rhodanilic acids did not. Ca, Ba and Cu′ salts yielded no insoluble products in water or aqueous alcohol. Ag′ partly precipitated the material, and Cu″ salts, prepared according to Town [1936], were less soluble in methyl alcohol than was the original material.

Properties (*d*) and (*e*) are interpreted as probably due to an amino group, and (*f*) and the Cu″ salt (*g*) to a carboxylic acid; the whole, including the separation 2–5 above, is consistent with the material being an amino-acid. The loss of one half of the activity with alkali may be due to racemization. Recombination of separated fractions, performed in each test, gave no indication of separation of the active material into two fractions of which both were necessary for reversal.

Marmite and the concentrates (5) were active in similar concentrations against streptococci inhibited by $8 \times 10^{-7} M$ euflavine, and marmite reversed the inhibitions of *Bact. typhosum* caused by $8 \times 10^{-6} M$, and of *Staphylococcus aureus* caused by $1{\cdot}6 \times 10^{-6} M$ euflavine.

The basal medium (p. 1311) already contains those amino-acids of greatest importance in bacterial nutrition. A further mixture of amino-acids, containing glycine, alanine, valine, leucine, *iso*leucine, lysine, arginine, histidine, proline, hydroxyproline, phenylalanine, tyrosine, aspartic and glutamic acids was active, with delay, as Type II factor (Table 3). Individually most of the acids were inactive or deleterious and the effect of the mixture was due mainly to phenylalanine. The properties of phenylalanine would probably cause it to be present in the active concentrates described above and phenylalanine acts in the Type II manner, but the concentrates were more active weight for weight than was this amino-acid. The following derivatives of phenylalanine were inactive or slightly active: tyrosine, 3:4-dihydroxyphenylalanine (delayed activity owing to removal of acriflavine from solution), thyronine, thyroxine. Many less related compounds, and also most of the substances described as promoting growth of organisms, had little or no reversing activity.

Further reversal and an interpretation of Types I and II activity

Throughout this work it had been borne in mind that acriflavine might act by interfering with structurally related compounds of importance to the inhibited organisms. Reversal with riboflavin and with phenazine derivatives [cf. Weil-Malherbe, 1937] was accordingly tried at several stages, but these were without activity under the conditions recorded in Tables 2 and 3. In the presence of different sources of Type II factor they were however immediately active (Table 4) in reversing $6 \times 10^{-5} M$ euflavine. No increase in the concentration of Type II factor brought about such reversal. This activity was however not at all specific, being given by methylene blue, brilliant cresyl blue, to some extent by phenosafranine, as well as riboflavin, pyocyanine and phenazine methosulphate (Table 4). As in animal nutrition, riboflavin as a bacterial growth

Table 4. *Hydrogen carriers in presence of Type II factors*

Special feature of media	Substance added	Molarity	Growth after: Hours 16–18	Hours 24	Days 2	Days 3
Marmite treated with norite,* 10 mg./ml.	0	—	0	0	0	0
	Pyocyanine or phenazine methosulphate	2×10^{-5}	+ + +	+ + + +	+ + + +	+ + + +
		4×10^{-6}	0	+ + +	+ + + +	+ + + +
	Riboflavin	10^{-4}	+ + +	+ + + +	+ + + +	+ + + +
	,,	2×10^{-5}	0	+ + +	+ + + +	+ + + +
Al_2O_3 eluate, 0·27 mg./ml.	0	—	0	0	0	0
	Pyocyanine	2×10^{-5}	+ + + +	+ + + +	+ + + +	+ + + +
	,,	4×10^{-6}	0	0	+ + + +	+ + + +
	Brilliant cresyl blue	5×10^{-5}	+ + +	+ + + +	+ + + +	+ + + +
	,,	5×10^{-6}	0	0	+ + + +	+ + + +
	Riboflavin	10^{-4}	0	+ +	+ + + +	+ + + +
	Uninhibited control on both media		+ + +	+ + + +	+ + + +	+ + + +

Inhibiting concentration of euflavine, $6 \times 10^{-5} M$.
* Marmite (25 g.) in water (to 125 ml.) stirred with norite (5 g.) at room temperature for 1 hr., filtered and neutralized.

factor is reported as being fairly specific [Snell & Strong, 1939], though attempts at replacement by the above hydrogen carriers are not recorded. This was accordingly tried by omission of riboflavin from the medium used for growth of *Streptococcus haemolyticus* and substitution of a wide range of concentrations of the above compounds. Growth did not occur, but it could be caused by further addition of $10^{-9}M$ and more of riboflavin, with groups A and C streptococci. Even 6:7-dimethylalloxazine was inactive. The above dyestuffs are thus not replacing riboflavin, but all, including riboflavin, may be replacing a hydrogen transporting system inactivated by the higher concentration of inhibitor.

These experiments, in which inhibited organisms can function only in the presence of such artificial redox systems and of a Type II factor, are reminiscent of many experiments with bacterial suspensions in which hydrogen carriers are found necessary for enzyme activity in the presence of added substrates. When such additions are necessary for growth of inhibited organisms, but not at all for normal ones, it is considered that the inhibited organisms lack both a substrate and a normal hydrogen transporting system. Lack of substrate (need for Type II factor) occurs first at a lower euflavine concentration ($3 \times 10^{-5}M$) and is presumably due to disturbance of the systems involved in its formation, since organisms such as *Bact. coli* do not normally need it pre-formed, and it is produced by many other normal cells. Interference with metabolizing enzymes, replaceable by the artificial carriers, occurs at euflavine concentrations of $6 \times 10^{-5}M$.

Acriflavine is known to inactivate many enzyme systems [Scheff & Hasskó, 1936; Quastel & Wheatley, 1931], and the previous experiments with nucleotides provide a mechanism for this interference. It is imagined that acriflavine forms complex salts with similar groupings essential to enzyme systems, and that the complexes are inactive. There was no simple relation between the inhibitory concentration of euflavine and the reversing concentration of pyocyanine. In an experiment in which $4 \times 10^{-6}M$ pyocyanine allowed growth in the presence of $6 \times 10^{-5}M$ euflavine, up to $2 \times 10^{-4}M$ pyocyanine gave a delayed effect only with $8 \times 10^{-5}M$ euflavine. This is interpreted as the inactivation of still further systems, which are pictured as being affected successively in order of stability of their different acriflavine complexes. Results with redox systems apply to *Bact. coli* but not to *Strep. haemolyticus*. Pyocyanine, and to a lesser degree riboflavin, contributed also to the reversal of inhibition of *Bact. coli* caused by proflavine, rivanol and crystal violet in the presence of marmite preparations.

Discussion

The results of the present studies of inhibitory action bear some fairly close relation to those which Fildes [1940, 1] obtained with mercuric compounds. These compounds are imagined to inhibit by combination with essential —SH groups in the cell, and can be reversed by added thiol compounds. No analysis of natural products which might lead to Type II reversal was carried out by him.

The removal of acriflavine inhibition by methylene blue etc. is not novel, but has previously been termed an example of chemotherapeutic interference [see Findlay, 1939], the interpretation of which has been realized as of importance in understanding the action of drugs. The present explanation does not necessitate combination of the interfering substance with the same group ('receptor') in the cell as combines with the inhibitory agent [see Wright & Hirschfelder, 1930], but rather the short-circuiting of such a group.

It has been asserted that the action of acriflavine is little affected by the growth medium, and is irreversible. Such views are no longer tenable. Thus,

normal bacteriological technique compares growth in peptone water, broth or serum media, but does not contrast growth in simple chemically defined solutions with that in others containing nucleate. Again, in metabolic experiments, reversal has been attempted by washing the inhibitor from the affected tissue, but not always by the addition of antagonistic substances. It is possible to 'resurrect' inocula of *Bact. coli*, rendered apparently non-viable on ordinary media by allowing them to stand for 30 min. in *M*/200 euflavine, with Type I or II factors.

A structural relationship between inhibitor and reversing agent, useful in modelling further therapeutic substances, has not yet emerged from this work, and the extent of the present knowledge of the enzymes and complex salts considered to be involved in acriflavine inhibition makes understandable the empiricism of current chemotherapy. Published work makes it probable that styryl 430 [Pourbaix, 1939] and germanin (Fourneau 309, Baeyer 205) [Oesterlin, 1935] act also by similar combination with enzyme systems, though the present technique of analysis of new nutrient needs has not yet been applied to them. Results with crystal violet and with rivanol have already been mentioned; in addition, marmite preparations and nucleate contributed to reversal of *Bact. coli* and *Bact. typhosum* inhibited by suitable concentrations of rivanol. The weakest step in the present arguments is the assumption that reversing agents characterized in the above manner are those normally involved in the blocked processes of the inhibited organisms. This is rendered more likely by using a variety of natural sources of the factors, and the assumption is one made in most straightforward nutritional work. At the least, the results would have significance as examples of 'detoxication mechanisms'; an instance of such a finding in animal nutrition is vitamin H.

Summary

A chemotherapeutic agent is regarded as depriving the inhibited organism of the use of enzymes or metabolites by various types of interference. The organism thus becomes nutritionally more exacting than in its normal state, and its new demands can, with due consideration for extraneous effects, be analysed by the usual techniques of bacterial nutrition.

Bact. coli and *Streptococcus haemolyticus*, inhibited by acriflavine components, required for further growth two types of material not normally needed. Type I (so called from a previous nutritional study of inhibitory action) was best replaced by nucleotides, and Type II by a concentrate of amino-acids, but partly by phenylalanine. In the presence of Type II compounds, but not without, artificial hydrogen carriers were further active against inhibition of *Bact. coli*. Type I compounds form complex salts with acriflavine components, and it is considered that the inhibitors inactivate enzyme systems of which Type I compounds are essential parts, of which Type II compounds are substrates or products, and of which some can be replaced by the hydrogen carriers.

I am greatly indebted to Mr D. E. Hughes for assistance throughout this work; to Dr F. Dickens for gifts of materials; and to several Departments of this University, and especially to Dr H. A. Krebs, for working facilities. The Marmite Food Extract Co., Ltd., kindly supplied yeast autolysate.

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148. STUDIES ON THE ACETONE-BUTYL ALCOHOL FERMENTATION

1. NUTRITIONAL AND OTHER FACTORS INVOLVED IN THE PREPARATION OF ACTIVE SUSPENSIONS OF *CL. ACETOBUTYLICUM* (WEIZMANN)

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This work was undertaken in order to devise a technique for studying the acetone butyl alcohol fermentation by *Cl. acetobutylicum* (Weizmann).[1] As in the case of other organisms of this group fermentation has hitherto been carried out in growth experiments in which carbohydrate was fermented in the presence of protein and other material (maize meal, glucose in yeast water etc.) and the fermentation products estimated during or at the end of the fermentation period.

Obviously this procedure does not readily lend itself to the study of the intermediate fermentative processes which would be facilitated if washed suspensions of the organism could be used in manometers and other small-scale apparatus. The fact that much work has been done on fermentation by this group, notably by the Wisconsin school, without the employment of this technique has been noteworthy, but is readily explained by the many pitfalls encountered in the attempts here recorded.

This work falls into two parts: (1) the discovery of a growth medium in which the organism ferments glucose so as to give a good yield of acetone and butyl alcohol; (2) the obtaining from such a medium of active cells which when separated from it are still capable of fermenting glucose with similar results.

Methods and technique

The media used are described in the course of the paper. The organism was maintained in the spore condition on sand; 60 ml. 5% maize meal in tubes 325×28 mm. were inoculated from this and incubated for 24 hr.; the medium, freshly autoclaved and quickly cooled, was inoculated from these tubes in the proportion of 2 ml. inoculum to 100 ml. medium and the flask was then filled with H_2 or N_2: incubation was at 37°. At the end of the fermentation the culture was examined microscopically the film being stained by acid carbolfuchsin.

The analytical methods employed were as follows. Glucose was estimated by Hanes's [1929] modification of the method of Hagedorn & Jensen as described by Widdowson [1931]; acetic and butyric acids and ethyl and butyl alcohols by the methods of Friedemann [1938]; lactic acid by the method of Friedemann & Kendall [1929]; acetylmethylcarbinol by the method of Langlykke & Peterson [1937], pyruvic acid by the carboxylase method of Westerkamp [1933].

Acetone was estimated by the following method using a lactic acid apparatus of the type described by Friedemann *et al.* [1927] but having ground glass joints throughout and a sintered glass filter in place of the absorption tower of glass

[1] We are indebted to Dr A. C. Thaysen of the Chemical Research Dept., Teddington, for the strain used and also for much kind help in methods of cultivation.

beads. An aliquot of culture medium containing 0·6–1·2 mg. acetone is introduced into the distilling flask and diluted to 30–40 ml. The receiving flask contains 25 ml. $N/100$ iodine + 5 ml. N NaOH [Bell & Harrison, 1939]. The condenser water is turned on and a fairly rapid stream of air is drawn through the apparatus; the contents of the distilling flask are rapidly brought to boiling point and boiled for 10 min.; boiling is then discontinued. The residual I_2 is liberated by the addition of 7 ml. N H_2SO_4 and titrated against $N/100$ $Na_2S_2O_3$. Blank estimations are made and the acetone is calculated from the I_2 disappearing by means of the equation:

$$CH_3COCH_3 + 3I_2 + 4NaOH \rightarrow CHI_3 + CH_3COONa + 3NaI + 3H_2O.$$

The method gives 98% recovery from standard acetone solutions. None of the fermentation products formed by *Cl. acetobutylicum* interferes with the determination appreciably.

On 5% maize meal *Cl. acetobutylicum* gives a fermentation in which acetone and butyl alcohol amount to about 8·5% and 23·4% respectively of the starch fermented. 2% glucose in a boiling water extract of yeast gives variable results; frequently the yields of solvents are very low and the fermentation is mainly an 'acid' one, whilst sometimes solvents appear and the fermentation approaches

Table 1. *Products of the fermentation of 2% glucose in yeast water by a growing culture of* Cl. acetobutylicum

	Exp. 1		Exp. 2	
Substance	mg./ml.	% glucose fermented	mg./ml.	% glucose fermented
Glucose initial	21·2	—	21·8	—
Glucose final	11·0	—	1·6	—
Glucose fermented	10·2	—	20·2	—
Acetone	0·77	0·75	1·10	5·46
Ethyl alcohol	0·58	0·57	0·362	1·79
Butyl alcohol	0·925	9·1	3·25	16·1
Acetic acid	0·716	7·0	1·085	5·38
Butyric acid	1·993	19·5	1·44	7·13

'normal' (see Table 1). A growth medium was therefore sought upon which glucose was fermented so as to give yields approximating to those obtained from the carbohydrate of maize meal.

Search for a growth medium

The basal medium was approximately that of McDaniel *et al.* [1939] based on Speakman's salt mixture cited by these authors; it had the following composition: 0·05% K_2HPO_4; 0·05% KH_2PO_4; 0·02% $MgSO_4, 7H_2O$; 0·001% $MnSO_4, 3H_2O$; 0·001% $FeSO_4, 7H_2O$; 0·001% NaCl; 0·1% asparagine; a tryptic digest of casein equivalent to 0·25% casein. The above was autoclaved at double strength and the requisite volume of 15% glucose, slightly acidified and separately autoclaved, was added to it; small pieces of filter paper were also present. To this, the 'basal medium', certain growth factor preparations were added as set out below, and the whole was diluted so as to contain 2% glucose.

Growth factor preparations

(1) *Weizmann's autolysate of yeast* [Weizmann & Rosenfeld, 1937]. 100 g. baker's yeast are plasmolysed with 10 ml. ethyl acetate, kept at *p*H 7·0 with 10% Na_3PO_4, toluene added and incubated at 37° for 48 hr. It is then

centrifuged and the supernatant liquid diluted to 400 ml.; 1 ml. is therefore equivalent to 0·25 g. fresh yeast.

(2) *Liver autolysate.* 500 g. fresh liver are passed through a Latapie mincer; 100 ml. *M*/15 phosphate buffer *p*H 7·4 are added and the whole diluted to 1 l. Toluene is added and the suspension is incubated for 7–10 days at 37°. It is then centrifuged and the supernatant used.

(3) *Tryptic digest of liver.* 500 g. fresh ox liver are passed through a Latapie mincer and suspended in 1 l. distilled water; 100 ml. pancreatic extract and 100 ml. *M*/15 Na_2HPO_4 are added and the *p*H adjusted to 8·0; toluene is added and the whole incubated for 7 days at 37°, the *p*H being kept at 8·0; the digest is then filtered and the filtrate concentrated *in vacuo* to 500 ml.; 1 ml. is therefore equivalent to 1 g. fresh liver.

(4) *Tryptic digest of maize meal.* 500 g. maize meal are treated as described above for tryptic digest of liver.

Testing the media

The basal medium, without glucose, was made up in double strength; 15 ml. of this, together with the required amount of growth factor preparations, were made up to 30 ml. and autoclaved for 45 min. at 15 lb. pressure; 3 ml. 15% glucose autoclaved separately in acid solution were then added. This was inoculated with 0·5 ml. of a 24 hr. culture of *Cl. acetobutylicum* on 5% maize meal and incubated at 37° in H_2. Growth was estimated by inspection after 15 and 39 hr. and the acetone in the media then estimated. The results are given in Table 2. It was found that the basal medium alone was inadequate for regular growth or acetone production. Any of the four preparations added produced

Table 2. *Effect of certain additions to the basal medium on growth and acetone production by* Cl. acetobutylicum

Source of growth factor	Amount added % calculated as equiv. amount of fresh substance	Growth				Acetone mg./ml. 39 hr.
		15 hr.		39 hr.		
		(*a*)	(*b*)	(*a*)	(*b*)	
Yeast autolysate	10	+ + +	+ + +	+ + +	+ + +	0·1
	5	+ + +	+ + +	+ + +	+ + +	0·02
	1	+ +	+ +	+ + +	+ + +	0·55
	0·25	+	+	+ + +	+ + +	0·59
	0·10	+	+	+ + +	+ + +	0·53
	0·05	(+)	(+)	+ + +	+ + +	0·28
Yeast water	10	?	(+)	+ + +	+ + +	0·16
	5	+ + +	+ + +	+ + +	+ + +	0·24
	1	+ +	+ +	+ + +	+ + +	0·45
	0·25	+	+	+ + +	+ + +	0·12
	0·10	+	+	+ + +	+ + +	0·09
	0·05	(+)	(+)	+ + +	+ + +	0·11
Tryptic digest of liver	10	+ + +	+ + +	+ + +	+ + +	0·10
	5	+ + +	+ + +	+ + +	+ + +	0·15
	2·5	+ + +	+ + +	+ + +	+ + +	0·68
	1·0	+ +	+ +	+ + +	+ + +	0·80
	0·5	+	+	+ + +	+ + +	0 90
	0·25	(+)	(+)	+ + +	+ +	0·48
Tryptic digest of maize meal	5	(+)	+ +	+ + +	+ + +	0·32
	2·5	+	+ +	+ + +	+ + +	1·03
	1·25	+ +	+	+ + +	+ + +	0·05
	0·5	(+)	0	+ +	+ +	0·03
	0·25	0	0	(+)	0	0
	0·10	0	0	+	0	0

good growth and good acetone yields. In each case there was an optimum concentration above and below which the acetone yield fell off. It is evident that the four preparations tested contain a growth factor or factors which may or may not be identical with another substance necessary for acetone production.

Table 3. *Comparison of growth and acetone production on a medium containing 0·1% asparagine and 0·1% $(NH_4)_2HPO_4$ respectively*

15 ml. double strength basal medium +3 ml. 1·0% asparagine or 3 ml. 1% $(NH_4)_2HPO_4$ +growth factor preparation+water to 30 ml.+3 ml. 20% glucose (separately autoclaved in acid sol.) inoculated with 0·5 ml. 24 hr. maize meal culture, filled with H_2 and incubated at 37°.

Growth factor	Equiv. amount of fresh substance %	Asparagine		$(NH_4)_2HPO_4$	
		Growth	Acetone mg./ml.	Growth	Acetone mg./ml.
Yeast autolysate	5·0	+ + +	0·143	+ + +	None
	1·0	+ + +	0·05	+ + +	None*
	0·25	+ + +	0·725	+ +	Trace
	0·1	+	0·07	+ +	Trace
Tryptic digest of maize meal	5·0	+ + +	0·143	+ + +	Trace
	2·5	+ + +	1·58	+ + +	None
	1·0	+ + +	Trace	+ + +	None
	0·5	+ +	Trace	+ +	None
Tryptic digest of liver	5·0	+ +	Trace	+ +	Trace
	2·5	+ +	0·591	+ +	Trace*
	0·5	+ +	0·247	+ +	None
	0·25	+ +	0·133	+	None

* These cultures gave positive test for pyruvic acid.

The replacement of asparagine in the basal medium by an equivalent amount of $(NH_4)_2HPO_4$ had no perceptible effect on growth but almost eliminated the production of acetone, the traces still found being probably due to asparagine

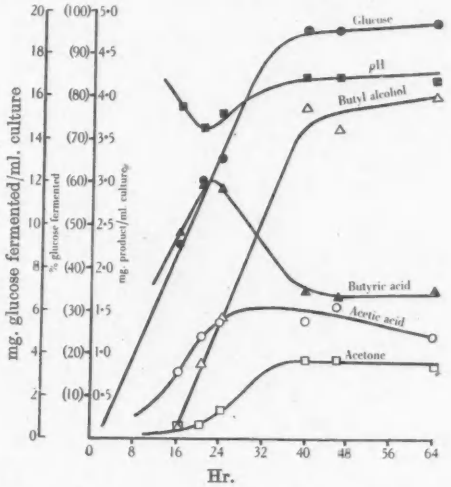

Fig. 1. Fermentation of glucose by *Cl. acetobutylicum*—course of reaction.

Table 4

Substrate	Medium	Product as % substrate (as sugar) fermented: Acetone	Butyl alcohol	Ethyl alcohol	Acetic acid	Butyric acid	Acetoin	CO_2	H_2	Formic acid	Lactic acid	Reference
1% glucose	0·5% peptone + salts	4·1	8·8	1·1	—	—	—	—	—	—	—	Johnson *et al.* [1931]
2% glucose	0·5% peptone + salts	8·3	20·5	2·5	—	—	—	—	—	—	—	Johnson *et al.* [1931]
4% glucose	0·5% peptone + salts	9·5	19·6	3·9	—	—	—	—	—	—	—	Johnson *et al.* [1931]
2·5% glucose	Yeast water	2·8	17·0	1·6	—	—	—	—	—	—	—	Langlykke *et al.* [1935]
2% glucose	Yeast water	3·4	20·2	—	10·3	10·5	—	51·7	1·75	Nil	Traces	Donker [1926]
4% maize meal	—	8·8	22·6	—	3·4	2·0	2·4	55·1	1·72	Nil	Traces	Donker [1926]
2% glucose	Yeast water	7·2	23·0	2·4	4·7	2·1	3·1	54·0	1·5	Traces	—	van der Lek [1930]
Maize meal* (industrial scale)	—	12·1	22·7	1·9	—	—	—	—	—	—	—	Speakman [1920]
6·5% maize meal* (1540 gallons of mash)	—	9·7	23·1	—	0·93	0·69	—	56·9	1·44	—	—	Reilly *et al.* [1920]
2% glucose	Tryptic digest of liver + salts + asparagine	7·9	23·8	Nil	5·73	6·75	—	—	—	—	—	Present authors
4·3% maize meal	—	8·5	23·4	0·45	2·2	1·44	4·1	—	—	—	Nil	Present authors
2% glucose in *M*/5 acetate	Tryptic digest of liver + salts + asparagine	14·2	21·2	0·2	—	—	—	—	—	—	—	Present authors
7·4% maize meal	—	14·2	26·4	3·0	0·71	1·55	—	—	—	0·08	—	Stiles *et al.* [1929]

* Yields recalculated from authors' figures to refer to % glucose fermented.

Table 5. *Effect of various substances on the production of gas from glucose by washed suspensions of* Cl. acetobutylicum

	Glucose	Pyruvate	Glucose and: Pyruvate	Filtered culture	Cell washing	Yeast autolysate	Glyceraldehyde	Glycerophosphate	Acetaldehyde	Formate	Asparagine	Glyceric acid	Methylene blue
Q_{gas}*	<1	24	34	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

* Q_{gas} = μl. total gas calculated as H_2/hr. and mg. dry wt. cells.

introduced with the growth factors,[1] see Table 3. In this connexion a possible parallel is found in the work of Tatum *et al.* [1935] and of Brown *et al.* [1938] who found in the case of the allied organism *Cl. butylicum* that asparagine added to the growth-fermentation maize-meal medium raised the production of neutral solvents, chiefly butyl alcohol, from 0·8 % to 19·9 % of the carbohydrate fermented. *Cl. acetobutylicum* shows no such effect with maize meal; possibly the latter organism possesses sufficient proteolytic activity to liberate asparagine from the maize proteins.

The medium finally adopted for growing the organism was the basal medium + tryptic digest of liver equivalent to 2·5 % fresh liver tissue. Growth on this medium produces a satisfactory amount of acetone and butyl alcohol and the course of the fermentation agreed with that obtained by other workers on this and related organisms (see Fig. 1 and Table 4).

The use of washed suspensions

In order to study the course of the fermentation it was desired to use manometric and other techniques involving washed suspensions of the organism. Great difficulty was encountered here owing to the fact that cells from an actively fermenting culture, when washed in *M*/15 phosphate buffer at *p*H 6·0, are generally inactive when put into glucose and buffer. This is the case whether the criterion of activity be reduction of methylene blue by glucose, gas production from or disappearance of glucose. Generally reduction of methylene blue was more active than gas production but the activity fell off rapidly. Finally it was decided to concentrate on obtaining a suspending solution in which the cells should show vigorous and sustained evolution of H_2 and CO_2 from glucose and pyruvate when tested in a manometer.

Warburg manometers were used each containing 1 ml. *M*/15 phosphate buffer *p*H 5·0; 1 ml. cell suspension (equivalent to 10 mg. dry wt./ml.) in the main vessel and 0·2 ml. *M*/10 glucose or *M*/15 pyruvate in one side bulb; the manometers were gassed with H_2 and shaken at 37°. Little or no gas production having been obtained in 30 experiments it was thought that some intermediate product might be needed as a 'starter' substance. The experiments were therefore repeated with the following substances in the second side bulb of the manometer; 0·2 ml. of one of the following: *M*/10 pyruvate; *M*/10 glyceraldehyde; *M*/10 Na glycerophosphate, *M*/10 acetaldehyde; *M*/10 Na formate; *M*/10 asparagine; 0·5 ml. filtered culture medium after growth and fermentation were complete; 0·5 ml. washings from cell suspensions; 0·5 ml. yeast autolysate; 0·5 ml. 0·1 % methylene blue. The results are given in Table 5; none of these substances was effective.

It was clear that in the culture medium the cells were active but that they lost their activity in the process of centrifuging, washing and resuspending in buffer. This was confirmed by centrifuging the culture and suspending the cells in the original culture fluid; the result was termed the 'concentrated culture'; 1 ml. of this used in a manometer gave a gas evolution of 350 μl. during 1 hr. owing to residual fermentable material. When this was over fresh glucose was tipped in from the side cup and a rapid gas evolution was obtained ($Q_{gas} = 258$, calc. as H_2). No gas evolution was obtained from a washed suspension of the same organisms.

The effect of suspending the centrifuged organisms in phosphate buffer without washing was then tried; this suspension produced no gas but was active

[1] In some preparations this may be sufficient to supply all the asparagine necessary.

in reducing methylene blue with glucose (see Table 6). A further increase in activity was obtained by de-oxygenating the suspending fluid by boiling, the $Q_{\text{methylene blue, glucose}}$ rising in one experiment from 33 to 216.

Table 6. *Effect of washing on the reduction of methylene blue by glucose and pyruvate using suspensions of* Cl. acetobutylicum

Each Thunberg tube contained 1 ml. phosphate buffer *p*H 5·0, 1 ml. *M*/10 glucose or *M*/10 pyruvate, 0·25 ml. 0·1% methylene blue. Temp. 37°.

	$Q_{\text{methylene blue}}$		
Substrate	Conc. culture	Unwashed suspension	Washed suspension
None	65	5	<1
Glucose	—	97	16
Pyruvate	—	70	37

The influence of various constituents of the culture medium in maintaining the activity of the culture was then tried, the activity of the 'concentrated culture' being the standard of comparison; Fig. 2 shows that all the constituents

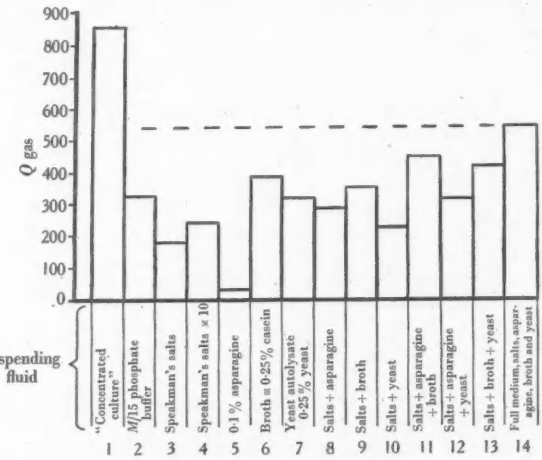

Fig. 2. Effect of suspending solution on unwashed cells as measured by gas production.

of the original medium contribute to the activity of the cells and that when the cells are resuspended in the original medium they retain 63% of their gas-producing activity. The gas production from pyruvate is less dependent on the suspending fluid, suspensions in solutions 2, 3, 8, 9, 10 and 14 giving a Q_{gas} between 222 and 266. In all subsequent experiments the cells were suspended in freshly boiled basal medium + yeast autolysate equivalent to 0·25% fresh yeast; the appropriate substrate being added from the side cup of the manometer vessel or as the experiment demanded. This medium is referred to as the Y.A. medium. The gas production from this medium without substrate is negligible and inactive suspensions are seldom obtained, provided that the suspension is used within a few minutes of its preparation as, even with these precautions,

decay is very rapid; for example, in the case of glucose the Q_{gas} fell from 400 to 18 in $2\frac{3}{4}$ hr.; that of pyruvate from 283 to 15 in 5 hr. A suitable growth medium and suspending fluid having been arrived at, preliminary experiments were now possible.

The p*H optimum for the fermentation of glucose and pyruvate*

The effect of *p*H on glucose fermentation was determined by three methods: (1) total gas production; (2) production of H_2; (3) disappearance of glucose. (1) and (2) were determined manometrically and (3) by the estimation of glucose. A preliminary difficulty was a choice of buffers to cover the range *p*H 3·5 to 7·5; the only single buffer available was McIlvaine's citrate-phosphate mixture [1921], but this inhibited the fermentation of glucose. Acetate buffers cover the range *p*H 3·5–6·0 but it was feared that, as acetic acid is a product of fermentation, this might have a disturbing influence on the fermentation; on testing the gas evolution at *p*H 5·0 in the presence of acetate and phosphate buffers respectively it was found that none occurred in the absence of glucose (or pyruvate) and that in the presence of substrate the rate with both buffers was identical; accordingly *M*/10 acetate was used for the range *p*H 3·5–6·0 and phosphate for the range *p*H 6·0–8·0.

For the manometric experiments each vessel contained 1·0 ml. cell suspension (about 5 mg. dry wt./ml.), and 1·8 ml. buffer (*M*/10 acetate or *M*/15 phosphate) in the main cup; 0·2 ml. *M*/10 glucose or pyruvate in the side bulb. In the case

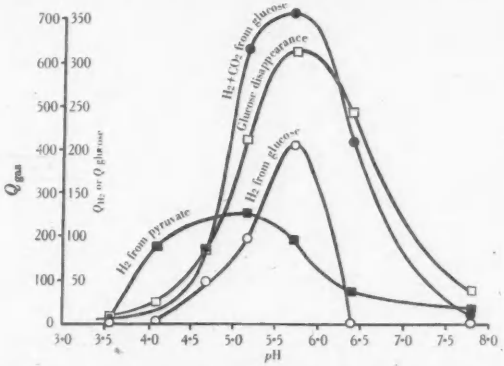

Fig. 3. *p*H-activity curves for fermentation of glucose and pyruvic acid by *Cl. acetobutylicum* (Weizmann).

of H_2 evolution 0·2 ml. 20 % NaOH and pleated filter paper were used in the centre cup; the vessels were gassed with H_2; the substrates were tipped into the main compartment after equilibration and the initial velocity of gas evolution over a period of 10 min. taken. The results are shown in Fig. 3.

Glucose disappearance was measured as follows: Krebs vessels were set up each containing 18 ml. buffer, 2 ml. *M*/10 glucose and 10 ml. cell suspension. H_2 was passed through the vessels for 5 min. and they were incubated at 38°. Samples were removed at intervals and glucose estimated. The glucose disappearing was plotted against time and the initial rate calculated; this was expressed as $Q_{glucose}$ (μl. glucose used/mg. dry wt. organism hr.; 1 m.-mol. $=22{,}400\,\mu$l.).

Fig. 3 shows that the optimum fermentation rate for glucose occurs at *p*H 5·7 irrespective of whether total gas evolution, H_2 evolution or glucose utilization is taken as the criterion. The optimum rate for pyruvate fermentation as measured by H_2 production was at *p*H 5·1.

Character of the fermentations of glucose and pyruvate by washed suspensions in Y.A. medium

In order to determine whether the fermentation by washed suspensions in Y.A. medium was an 'acid' or a 'normal' one the following products were estimated: acetone, butyl alcohol, acetic and butyric acids, acetoin, lactic acid, residual substrate, CO_2 and H_2.

The organisms were grown in the usual way, centrifuged and suspended in Y.A. medium; the fermentations were done in Krebs vessels; H_2 and CO_2 were measured manometrically each manometer containing 1/40th of the volume

Table 7. *Products of fermentation of glucose by cell suspensions of* Cl. acetobutylicum

Krebs vessels contained 30 ml. *M*/15 phosphate buffer *p*H 5·12, 10 ml. *M*/10 glucose, 20 ml. cell suspension 9·5 mg. dry wt./ml.; gassed with H_2; temp. 37°. The fermentation was complete in 4 hr.

	Product mg.	Product as % glucose fermented	C in product mg.	H in product mg.	O in product mg.
Initial glucose	360	—	—	—	—
Final glucose	33·3	—	—	—	—
Glucose fermented	326·7	—	130·7	21·8	174
H_2	6·96	2·13	—	6·96	—
CO_2	156·5	48	42·7	—	113·8
Acetic acid	36·0	11·0	14·4	2·24	19·2
Butyric acid	107·6	32·9	58·7	9·79	33·8
Acetone	4·9	1·5	3·0	0·57	1·4
Butyl alcohol	None	—	—	—	—
Ethyl alcohol	"	—	—	—	—
Pyruvic acid	"	—	—	—	—
Lactic acid	"	—	—	—	—
Acetoin	"	—	—	—	—
Total C, H and O in products			118·8	19·5	168·2

Table 8. *Products of fermentation of pyruvic acid by cell suspensions of* Cl. acetobutylicum

Krebs vessels contained 20 ml. *M*/15 phosphate buffer *p*H 4·86, 20 ml. cell suspension 23 mg. dry wt./ml.; 20 ml. *M*/20 pyruvate; gassed with H_2; temp. 37°. Fermentation complete in 4 hr.

	Total product mg.	Product as % pyruvate fermented	C in product mg.	H in product mg.	O in product mg.
Initial pyruvic acid	155·5	—	—	—	—
Final pyruvic acid	None	—	—	—	—
Pyruvic acid fermented	155·5	—	63·62	7·06	85·00
H_2	2·24	1·44	—	2·24	—
CO_2	60·3	38·8	16·46	—	43·85
Acetic acid	80·2	51·6	32·08	5·31	42·70
Butyric acid	19·15	12·3	10·41	1·74	6·97
Acetone	None	—	—	—	—
Ethyl alcohol	"	—	—	—	—
Butyl alcohol	"	—	—	—	—
Acetoin	"	—	—	—	—
Lactic acid	"	—	—	—	—
Total C, H or O			59·95	9·29	93·52

used in the Krebs vessel; the quantities used are given in Tables 7 and 8; the manometric experiment was run concurrently with the main experiment and served also to show when the reaction reached a standstill. 30 min. after the gas evolution in the manometers had ceased the contents of the Krebs vessels were centrifuged and the deposit of organisms washed once; the supernatant and washings were made up to 70 ml. and analysed. It is clear from Tables 7 and 8 that the fermentation occurring is essentially an acid one, acetone being absent in the case of pyruvate and present in low amounts only from glucose. (In our experience the production of acetone and the alcohols from glucose are so closely related that for exploratory experiments the former only need be estimated.) The cause of the absence of solvents was next investigated.

The effect of the age of the culture on the character of the fermentation

A fermentation of glucose in 5 l. of the usual glucose-liver medium was carried out. The 'age' of this culture was checked by glucose determinations, *p*H measurements and acetone determinations. Samples were withdrawn at intervals and used for the preparation of suspensions in Y.A. medium. The fermenting power of these suspensions was tested as follows: 7 ml. cell suspension, 4·2 ml. 2 *M* acetate buffer at *p*H 5·0, 1 ml. *M* glucose, 1·4 ml. 1·5 % asparagine and 7·4 ml. water were put into a Thunberg tube modified so as to permit of gassing with H_2. After 20 hr. glucose disappearance and acetone production were measured. The results are given in Tables 9 and 10.

Table 9. *Effect of age of culture on acetone produced (a) in the culture medium, (b) by cell suspensions prepared from it*

Age of culture hr.	Growth mg./ml. dry wt.	% glucose fermented	Acetone in culture mg./ml.	Acetone formed by cell suspensions mg./ml.
6	0·291	5	None	None
8	0·965	11·8	None	None
10	1·30	25·3	0·038	0·09
12	1·34	33·9	0·09	0·154
14	1·30	38·0	0·123	0·190
17	1·24	40·2	0·163	0·111
22	1·35	41·6	0·181	0·091
24	1·085	44·8	0·193	0·251
43	0·695	71·5	0·707	0·082

Table 10. *Effect of the age of the culture on the activity of the suspensions of* Cl. acetobutylicum

Age of culture hr.	Growth mg./ml. dry wt.	% glucose fermented	Acetone in culture mg./ml.	Acetone formed by cell suspension mg./ml.
13	1·36	34·0	0·137	0·594
16	1·40	47·5	0·298	—
19	1·38	56·7	0·493	2·10
22	1·32	65·8	0·653	2·09
25	1·19	74·9	1·106	1·91
40	—	95·4	1·15	0·051

In Table 9 it is seen that up to 8 hr. no acetone occurred in the parent culture neither was acetone produced by the corresponding suspensions. From 10 hr. onwards acetone appears in the parent culture and is also formed by the suspensions. The activity of the suspension in respect of acetone production increases with increasing amounts of acetone in the parent culture up to a maximum, after

which it remains fairly constant for some hours. Without emphasizing the quantitative aspect of these experiments one deduces that in the early stage of the fermentation some essential part of the enzyme complex necessary for acetone production is absent; not until acetone is present in the parent culture is the corresponding suspension capable of producing it. Hence, when investigating acetone production by cell suspensions, it is necessary to harvest the cells after the appearance of acetone in the culture medium; such suspensions will hereafter be referred to as 'ripe' suspensions.

Fermentation by suspensions of ripe organisms

The organisms were grown on the usual medium and after the appearance of acetone the cells were harvested and suspended in Y.A. medium. The suspensions were incubated with $M/10$ glucose in an atmosphere of H_2 and analyses made

Table 11. *Fermentation of glucose in Y.A. medium*

Krebs vessel contained 8 ml. *M* glucose, 22 ml. Y.A. medium and 50 ml. cell suspension (26·4 mg./ml. dry wt.). The medium was inoculated as usual and gassed with H_2. Samples were withdrawn at 6 hr. and 22 hr. for analysis and determination of *p*H.

	Initial	6 hr.	22 hr.	Products as % glucose fermented
Glucose, mg./ml.	20·0	1·23	1·07	—
*p*H	5·97	4·40	4·47	—
Acetone, mg./ml.	—	0·87	0·81	4·5
Butyl alcohol, mg./ml.	—	3·5	3·6	18·9
Butyric acid, mg./ml.	—	—	2·57	13·6
Acetic acid, mg./ml.	—	—	1·47	7·8

after 6 and 22 hr. The results are given in Table 11. This shows that a good production of solvents can be obtained by suspensions of the organisms if harvested at the right time and suspended in an adequate medium.

Summary

1. The fermentation of carbohydrate by *Cl. acetobutylicum* depends on the presence of a number of factors besides the organism and the carbohydrate.

2. On 5% maize meal the organism grows readily and ferments the carbohydrate of the maize, producing CO_2, H_2, acetic and butyric acids, ethyl and butyl alcohols and acetone ('normal fermentation'). On 2% glucose and yeast water the yields of alcohols and acetone are very low ('acid fermentation').

3. In order to convert an acid fermentation into a normal fermentation certain additions are necessary; these have not yet been isolated but are present in liver, maize and yeast and can be extracted from these by suitable procedures. There is an optimal amount of each of these extracts above and below which the yield of acetone falls off.

4. Cells grown on a basal medium + liver extract give good growth and solvent production but when spun off and washed are totally inactive.

5. A suspending fluid in which the centrifuged cells retain their fermentative activity was at length found by the use of which the course of the fermentations of glucose and pyruvate have been studied.

6. The character of the fermentation depends on the age of the culture from which the cells are harvested; those obtained in the early stages of the fermentation produce no acetone in subsequent fermentations; only if harvested after the appearance of acetone in the parent culture are cells obtained which later produce a normal fermentation. Such organisms are termed 'ripe'.

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149. THE INFLUENCE OF HALIDES ON THE OXIDATION OF ASCORBIC ACID

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Past work on the influence of inorganic salts on the stability of ascorbic acid is somewhat conflicting. De Caro & Giani [1934] reported that the salts of Ringer's solution had a stabilizing effect on ascorbic acid; Kellie & Zilva [1935] noted that sodium, potassium and calcium chlorides decreased the oxidation of the vitamin at 37° and considered that they acted by inhibiting the catalytic action of copper. Barron *et al.* [1936], however, could not confirm the inhibiting effect of NaCl on the catalytic oxidation of ascorbic acid by copper at a *p*H of 6·00 in a phosphate buffer.

Klodt & Stieb [1938] found that the addition of NaCl to orange juice increased the stability. Høygaard & Rasmussen [1938] reported less destruction of the vitamin when potatoes, cabbage and liver were cooked in water containing NaCl than when they were cooked in distilled water; the increased retention of the vitamin due to the addition of the salt was, however, in no case greater than 19%, and with liver the effect of the salt was negligible.

The evidence from these studies is therefore confusing, and still less clear are the conditions under which such a salt as NaCl acts. Finally, it has not been shown conclusively which ion of NaCl is active, although Kellie and Zilva's work implied that it was the halide ion, since they obtained positive results with calcium, potassium and sodium chlorides.

The present paper deals with a more detailed investigation into the influence of NaCl and related salts. The results indicate clearly that the catalytic oxidation by Cu is either augmented or diminished, depending on conditions, by chloride, bromide or iodide ions.

Experimental

Determinations of ascorbic acid were carried out chemically by the visual method of Harris & Ray [1933] and Birch *et al.* [1933] using 2:6-dichlorophenol-indophenol. Before titration, all solutions were made up to volume with sufficient 20% metaphosphoric acid to produce a final concentration of 5%. When iodides were used ascorbic acid was determined by titration with iodine. *p*H was determined electrometrically with the quinhydrone electrode.

All solutions were made up with water freshly distilled from a pyrex glass still, and unless otherwise stated, the water was aerated thoroughly before each experiment to ensure that it was fully saturated with air. This procedure was essential, for if freshly boiled distilled water was used, the oxidation of ascorbic acid was much reduced.

Ascorbic acid was added initially to all solutions in a concentration of 10 mg./100 ml. of solution. All reagents used were of analytical purity.

Part I. Experiments at 100°

The destruction of ascorbic acid in acid solutions heated to 100° was due entirely to oxidation, since it was prevented if the O_2 was completely removed beforehand. Thus ascorbic acid in the presence of the weak organic acids, citric, malic, tartaric or acetic, was not oxidized if N_2 or CO_2 were bubbled through the solution for 15–20 min. before heating. The losses of ascorbic acid in the experiments recorded in this paper were therefore caused by oxidation, and not by any effect of heat *per se* on the acid. Ascorbic acid was relatively stable in solutions of citric, malic, tartaric or acetic acids at room temperature in the presence of O_2, but the rate of destruction increased rapidly with rise of temperature.

The rate of oxidation of pure ascorbic acid in a solution of 0·1 *M* acetic acid heated at 100° is shown in Fig. 1. It proceeded rapidly for the first 30 min.,

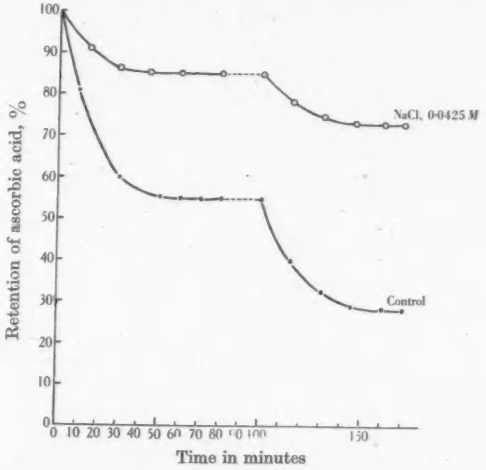

Fig. 1. Oxidation of ascorbic acid in 0·1 *M* acetic acid at 100° and *p*H 2·87.
----- Solution cooled to room temperature and aerated.

and then virtually ceased. This was due to the elimination of O_2 from the heated solution, for if the latter were cooled to room temperature, re-aerated and then reheated, more ascorbic acid was oxidized, until all the O_2 had again been driven out of the solution (see Fig. 1). The stability of ascorbic acid at 100° has accordingly been determined by heating for 30 min. solutions of the acid in flasks fitted with steam condensers and immersed in a boiling water bath. This period of heating was selected as the longest that could be employed before lack of O_2 brought the reaction to an end. After heating, the flasks were cooled quickly to room temperature and their contents made up to a measured volume with metaphosphoric acid.

When NaCl was added to solutions of ascorbic acid in citric, tartaric, malic, acetic or sulphuric acids, the rate of oxidation of the acid was depressed in comparison with that of control solutions in which no salt was present (see Fig. 1). The protective influence of the NaCl increased with the amount of salt

added, provided that the *p*H of the solution was not more acid than 2·0 or less acid than 6·0. Typical results showing this influence of NaCl are given in Table 1. Control solutions, to which NaCl was added without ascorbic acid, were heated for 30 min. at 100° and did not reduce the indophenol dye. The addition of salt in the concentrations used did not significantly alter the *p*H (Table 1). In view

Table 1

In each instance the solution originally contained 10 mg. ascorbic acid/100 ml.

Solution	Molarity of added NaCl	*p*H	Ascorbic acid after heating 30 min. at 100° mg./100 ml.	% destruction of ascorbic acid	Ascorbic acid retained in solution with salt compared with control =100
1% citric acid	—	2·35	6·50	35	100
	0·021	2·36	7·90	21	122
	0·042	2·34	8·50	15	131
	0·085	2·34	9·10	9	140
	0·170	2·33	9·60	4	148
	0·250	2·33	9·70	3	150
	0·50	2·32	9·80	2	151
1% tartaric acid	—	2·30	6·40	36	100
	0·042	2·29	7·60	24	119
	0·085	2·30	7·90	21	123
	0·170	2·28	8·50	15	133
	0·250	2·28	9·10	9	142
	0·50	2·27	9·20	8	144
1% malic acid	—	2·47	6·80	32	100
	0·042	2·46	8·20	18	142
	0·085	2·46	8·50	15	146
	0·170	2·45	9·10	9	134
0·1 *N* acetic acid	—	2·87	6·50	35	100
	0·042	2·87	9·25	7·25	142
	0·085	2·87	9·50	5	146
	0·170	2·88	9·90	1	153
	0·250	2·87	10·00	Nil	154
	0·50	2·86	10·00	Nil	154
0·1 *N*. H_2SO_4 (adjusted with NaOH to *p*H 2·30)	—	2·30	4·20	58	100
	0·042	2·30	8·80	12	210
	0·085	2·30	9·30	7	221
	0·170	2·29	9·40	6	225
	0·250	2·28	9·50	5	226
	0·50	2·27	9·70	3	231

of possible practical applications attention has been chiefly directed to the three fruit acids, citric, tartaric and malic, but the investigation has also included such acids as acetic, glycollic, succinic and sulphuric. The same general results have, however, been obtained with all the acids so far examined, differences between them being of a quantitative, not qualitative nature.

Many inorganic salts have now been tested in the same manner to determine their influence on the oxidation of ascorbic acid. In most cases the *p*H of the solutions was not significantly altered by the addition of the various salts. When any significant change in *p*H would have been introduced by the addition of the salt, a mixture of salt and acid of the salt was added. With sodium fluoride, the *p*H was maintained constant by adjustment with H_2SO_4.

Typical results are shown in Table 2. Of all the salts tested, only those containing chloride, bromide or iodide ions showed any inhibitory influence on the oxidation of ascorbic acid. Sulphates, nitrates, acetates and phosphates

Table 2

In each instance the solution originally contained 10 mg. ascorbic acid/100 ml.

Solution	Molarity of salt added	*p*H	Ascorbic acid after heating 30 min. at 100° mg./100 ml.	% destruction of ascorbic acid	Ascorbic acid retained in solution with salt compared with control = 100
	NaH_2PO_4				
1% malic acid	—	2·47	5·90	41	100
	0·042	2·60	4·60	54	78
	0·085	2·73	4·25	57·5	72
	0·170	2·93	3·70	63	63
	NaH_2PO_4-H_3PO_4				
1% malic acid	—	2·47	6·20	38	100
	0·04	2·47	6·08	40	97
	0·08	2·47	5·90	41	95
	0·17	2·47	5·80	42	93·5
	0·34	2·47	5·75	42·5	92·5
	Na_2SO_4				
1% malic acid	—	2·46	6·30	37	100
	0·042	2·46	5·50	45	87
	0·085	2·50	5·40	46	86
	0·170	2·54	5·60	44	89
	$CaSO_4$				
1% malic acid	—	2·46	5·90	41	100
	0·010	2·47	5·36	46	91
	0·020	2·48	4·95	50·5	84
	Acetic acid				
1% malic acid	—	2·47	6·10	39	100
	0·042	2·44	6·00	40	98·5
	0·085	2·41	6·05	39·5	99
	0·170	2·38	6·10	39	100
	$(NH_4)NO_3$				
1% malic acid	—	2·46	6·00	40	100
	0·042	2·46	6·00	40	100
	0·085	2·46	5·90	41	98
	0·170	2·46	5·80	42	97
	0·250	2·46	5·70	43	96
	$(NH_4)_2SO_4$				
1% malic acid	—	2·46	6·20	38	100
	0·042	2·48	6·00	40	97
	0·085	2·50	5·90	41	95
	0·170	2·57	6·00	40	97
	0·340	2·58	5·90	41	95
	KBr				
1% malic acid	—	2·47	6·40	36	100
	0·042	2·47	9·10	9	142
	0·085	2·48	9·60	4	150
	0·170	2·49	10·00	0	157
	KCl				
1% malic acid	—	2·47	5·90	41	100
	0·021	2·46	7·40	26·0	125
	0·042	2·47	7·85	21·5	133
	0·085	2·48	8·40	16	143
	0·170	2·45	8·70	13	148
	0·340	2·45	8·80	12	149
	$MgCl_2$				
1% malic acid	—	2·46	5·90	41	100
	0·021	2·46	7·60	24	129
	0·042	2·45	8·70	13	147
	0·085	2·42	9·30	7	157

Table 2 (*cont.*)

Solution	Molarity of salt added	*p*H	Ascorbic acid after heating 30 min. at 100° mg./100 ml.	% destruction of ascorbic acid	Ascorbic acid retained in solution with salt compared with control =100
	NaF				
1% malic acid	—	2·53	6·20	38	100
	0·021	2·54	6·10	39	98·5
	0·042	2·57	5·50	45	89
	0·085	2·52	5·20	48	84
	0·170	2·51	4·50	55	73
	$BaCl_2$				
1% malic acid	—	2·47	6·10	39	100
	0·021	2·47	7·60	24	125
	0·042	2·47	8·60	14	141
	0·085	2·44	9·10	9	150
	$CaCl_2$				
1% tartaric acid	—	2·30	6·40	36	100
	0·021	2·28	7·80	22	122
	0·042	2·26	8·40	16	132
	0·085	2·22	9·40	6	148
	KI				
1% citric acid	—	2·35	6·50	35	100
	0·021	2·49	8·70	13	134
	0·042	2·63	9·00	10	139
	0·085	2·75	9·20	8	142
	0·170	2·95	9·40	6	145
	0·340	3·13	9·60	3	148

either had no effect or had a somewhat deleterious influence. Fluorides, it will be noted, in a concentration varying from 0·02 to 0·34 *M* afforded no protection in any of the acid solutions examined.

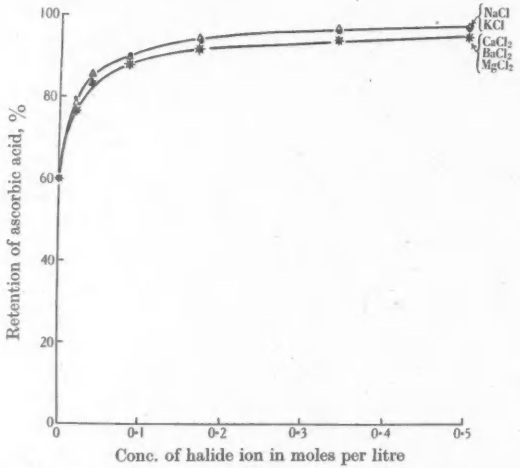

Fig. 2. Comparison of effects of various chlorides on oxidation of ascorbic acid. Solution: 1% citric acid, *p*H 2·35, heated 30 min. at 100°.

The results demonstrate that the effective ion is the halide ion, and this supposition is confirmed when quantitative comparisons are made of the effi-

ciency with which the chlorides of calcium, barium, magnesium, potassium and sodium protect ascorbic acid when added in concentrations equivalent to their content of halide. The effectiveness of salts of the type MCl and MCl_2 in inhibiting the oxidation of ascorbic acid in a solution of 1 % citric acid, *p*H 2·35, is shown in Fig. 2. The degree of protection obtained depends on the concentration of the halide ion added, and is independent of the anion of the salt. In concentrations exceeding 0·025 *N* halide the protective influence of salts of the type MCl_2 is somewhat less than salts of the type MCl.

Influence of copper. In the results so far recorded reagents of the usual analytical purity were used. These, however, invariably contain traces of heavy metals such as Cu. Barron *et al.* [1936] have shown that ascorbic acid is not autoxidizable in pure solution, but reacts with O_2 only when Cu is present as a catalyst. They found that at 25° Cu at a concentration of 1 part in 20 million (46 μg./l.) was still sufficient to catalyse the oxidation at *p*H 3·17 in a citrate buffer.

The amounts of Cu present in the reagents used in the present experiments were determined with sodium diethyl-dithio-carbamate according to the method of Callan & Henderson [1929]. As modified by Eden & Green [1940], the reagent is capable of detecting Cu at a dilution of 1 part in 100 million.

The glacial acetic acid contained 1·7 parts of Cu in 10^6, the citric, tartaric and malic acids 1–1·5 parts in 10^6 and the glass distilled water no detectable amount. The ascorbic acid contained 1 part per million, but owing to the very small amount added, 10 mg./100 ml., the amount of Cu thus introduced into the solution was insignificant (1 part in 10^{10}).

The main sources of Cu were thus the acids. In the final concentrations the amount of Cu present was 1 part in 100×10^6 for 0·1 *M* acetic acid; for tartaric, citric and malic acids 1 part in 70×10^6. These concentrations, small though they are, were still sufficient to cause catalytic oxidation of ascorbic acid, as the following experiment shows.

Glacial acetic acid was distilled in an all-glass Pyrex apparatus and made up in a concentration of 0·1 *M*, ascorbic acid added and the stability of the latter tested by the routine procedure. An estimation of the Cu present in the distilled acid showed that it had been reduced to approximately 1 part in 4×10^6, giving a final concentration in the solutions used for the test of 1 part in 650×10^6. With this amount, the loss of ascorbic acid due to oxidation was negligible—in one experiment there was a loss of 3%, in another there was no loss.

By the addition of known small amounts of Cu to the redistilled acetic acid, the amount of Cu necessary to cause oxidation of ascorbic acid under the standard conditions could be ascertained. These points are illustrated in Table 2A.

Table 2A

In each instance the solution originally contained 10 mg. ascorbic acid/100 ml.

Solution	Cu present parts/million	Ascorbic acid after heating 30 min. at 100° mg./100 ml.	% destruction of ascorbic acid
0·1 *M* acetic acid (glass distilled)	0·0015	9·7	3
"	0·0015	10·0	0
"	0·0033	9·0	10
"	0·01	7·5	25
"	0·05	6·0	40
"	0·10	3·6	64
"	0·5	1·6	84
"	2·5	0·5	95

A concentration of more than 1 part of Cu in 650×10^6 must therefore be avoided if ascorbic acid is to be completely protected against oxidation at 100°.

Attempts were made to eliminate Cu from the other acids studied, but results have not been entirely successful. Citric acid, even after four or five recrystallizations, still contained 1 part in 2×10^6, and when made up in a 1% solution gave a concentration of 1 part in 200×10^6. Tests with this citric acid showed that, as the concentration of Cu was reduced, less ascorbic acid was lost (Table 2B).

Table 2B

In both instances the solution originally contained 10 mg. ascorbic acid/100 ml.

Solution	Cu present parts/million	Ascorbic acid after heating 30 min. at 100° mg./100 ml.	% destruction of ascorbic acid
1% citric acid	0·014	6·0	40
1% citric acid (4th recrystallization)	0·005	7·5	25

As mentioned in the introduction, Kellie & Zilva [1935] found that the oxidation of ascorbic acid in solutions to which Cu was added was much less if NaCl were present. Cu salts easily form complex salts with the halogens, and this offers the simplest explanation of the protective action of the halide ions.

To test this hypothesis various amounts of Cu as $CuSO_4$ were added to different acid solutions, and the stability of ascorbic acid at 100° was determined in the presence of added halide salts in the usual manner. Experiments in which acetic and citric acid solutions were used, which are typical of the general results obtained, are illustrated in Figs. 3 and 4, respectively. The acetic acid was freed from Cu by distillation, and allowance was made for the Cu present as impurity in the citric acid. It is clear that the halide ion, if added in sufficient concentration, inhibits the catalytic oxidation of ascorbic acid by Cu, and that the extent of this inhibition depends on the actual concentration of Cu present; the greater the concentration of Cu, the greater the concentration of the halide necessary to obtain the same protection. To suppress entirely the oxidation of ascorbic acid in 0·1 *M* acetic acid at *p*H 2·87 with Cu present in the following concentrations, $0{\cdot}12 \times 10^{-7}$, 5×10^{-7} and 25×10^{-7}, chloride must be present in concentrations of 0·2, 0·7 and 1·6 *M*, respectively. With corresponding amounts of Cu, more NaCl was needed to suppress the oxidation to an equivalent extent in the citric acid, than in the acetic acid solution.

When chloride was present in very low concentration it had a small but definite deleterious influence on the stability of ascorbic acid. The range of concentration of chloride throughout which this slight destructive influence was observed, increased as the content of Cu present in solution was increased. For example, under the conditions shown in Fig. 3, with Cu present in a concentration of $0{\cdot}12 \times 10^{-7}$, NaCl had to be added in excess of 0·001 *M* to suppress the oxidation below that in the control solution, and the range of salt throughout which the destructive influence was found was 0–0·001 *M*. With Cu in a concentration of 25×10^{-7}, on the other hand, it was necessary to have the halide present in a concentration of more than 0·02 *M* before any protection was obtained, and the range of concentration in which the destructive effect was observed correspondingly increased.

Relative efficiencies of chloride, bromide and iodide ions at 100°. The efficiency with which the three halide ions protect ascorbic acid from oxidation at 100° has been determined in the range of concentrations of 0·0001–0·5 *M*. Neither

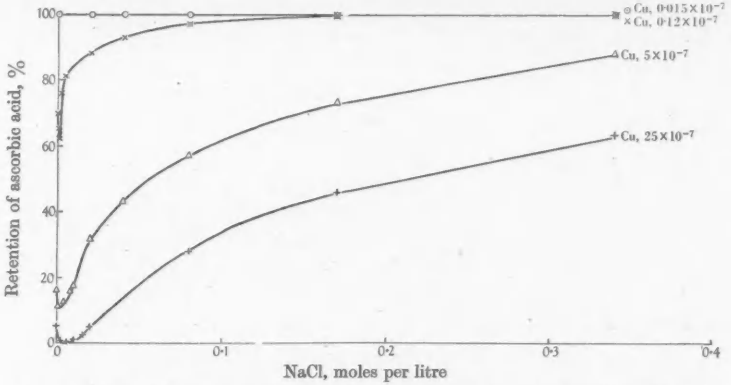

Fig. 3. Relation between concentration of Cu and chloride in oxidation of ascorbic acid. Solution: 0·1 *M* acetic acid, *p*H 2·87, heated 30 min. at 100°.

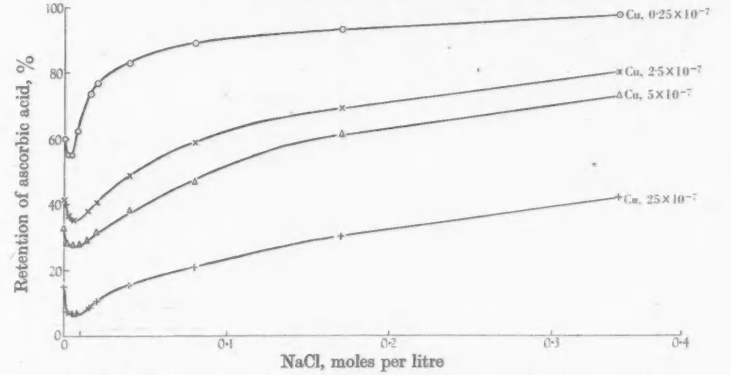

Fig. 4. Oxidation of ascorbic acid as influenced by Cu and halide, in solution of citric acid, 1%. *p*H 2·35, heated 30 min. at 100°.

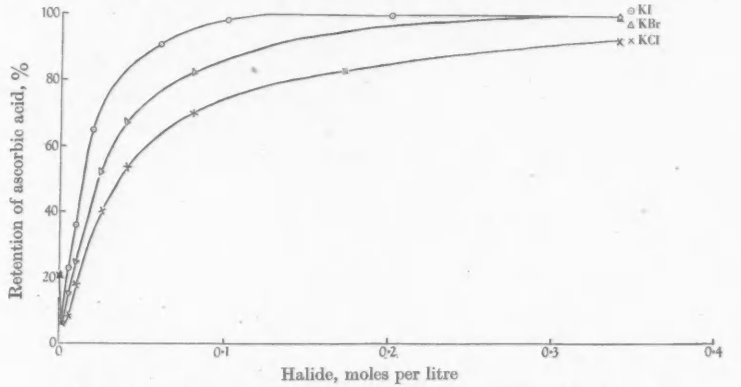

Fig. 5. Comparison of effects of various halides on oxidation of ascorbic acid. Solution: 0·1 *M* acetic acid, *p*H 2·87, Cu = 5×10^{-7}, heated 30 min. at 100°.

Table 3

In each instance the solution originally contained 10 mg. ascorbic acid/100 ml.

Solution	Molarity of salt added	*p*H	Ascorbic acid after heating 30 min. at 100° mg./100 ml.	% destruction of ascorbic acid	Ascorbic acid retained in solution with salt compared with control =100
	KCl				
1% malic acid	—	2·43	6·00	40	100
	0·02	2·43	7·40	26	123
	0·04	2·43	7·85	21·5	131
	0·08	2·43	8·40	16	140
	0·17	2·43	8·70	13	135
	0·34	2·43	8·90	11	149
	KBr				
1% malic acid	—	2·43	6·20	38	100
	0·02	2·43	8·20	18	132
	0·04	2·43	9·10	9	147
	0·08	2·43	9·60	4	155
	0·17	2·43	10·00	0	162
	KI				
1% malic acid	—	2·43	6·20	38	100
	0·02	2·43	9·10	9	147
	0·04	2·43	9·60	4	155
	0·08	2·43	9·90	1	160
	KCl				
1% tartaric acid	—	2·25	6·40	36	100
	0·02	2·25	6·85	31·5	107
	0·04	2·25	7·25	27·5	113
	0·08	2·25	7·90	21	124
	0·17	2·25	8·45	15·5	132
	KBr				
1% tartaric acid	—	2·25	6·40	36	100
	0·02	2·25	8·10	19	127
	0·04	2·25	8·60	14	135
	0·08	2·25	8·90	11	139
	0·17	2·25	9·10	9	143
	0·34	2·25	9·30	7	146
	KI				
1% tartaric acid	—	2·25	6·40	36	100
	0·02	2·25	8·90	11	139
	0·04	2·25	9·40	6	147
	0·08	2·25	9·60	4	150
	KCl				
1% citric acid	—	2·35	5·90	41	100
	0·02	2·35	7·80	22	132
	0·04	2·35	8·50	15	144
	0·08	2·35	9·00	10	152
	0·17	2·35	9·50	5	161
	0·34	2·35	9·70	3	165
	KBr				
1% citric acid	—	2·35	5·90	41	100
	0·02	2·35	8·20	18	139
	0·04	2·35	9·00	10	152
	0·08	2·35	9·30	7	158
	0·17	2·35	9·60	4	163
	0·34	2·35	9·90	1	168
	KI				
1% citric acid	—	2·35	5·90	41	100
	0·02	2·35	9·10	8	155
	0·04	2·35	9·30	6	158
	0·08	2·35	9·50	4	161
	0·17	2·35	9·70	2	164
	0·34	2·35	10·00	0	169

KCl nor KBr when added in these concentrations to the acid solutions examined caused any appreciable change in *p*H, but KI, in the highest concentrations used, altered it slightly; when this was so the *p*H was readjusted with dilute H_2SO_4. The relative influence of these ions in amounts varying from 0·001 to 0·34 *M* on the oxidation of ascorbic acid in a solution of 0·1 *M* acetic acid, *p*H 2·87, with Cu present at a concentration of $2·5 \times 10^{-7}$, is illustrated in Fig. 5, and further examples with other acid solutions are shown in Table 3. The order of efficiency of the ions is chloride < bromide < iodide. Some typical results obtained with weaker solutions than 0·02 *M* of halide are shown in Table 4.

Table 4

In each instance the solution originally contained 10 mg. ascorbic acid/100 ml.

Solution	Molarity of salt added	Concentration of Cu present	Ascorbic acid after heating 30 min. at 100° mg./100 ml.	% destruction of ascorbic acid
	KCl			
0·1 *M* CH_3COOH, *p*H 2·87	—	5×10^{-7}	1·8	82
	0·0001	5×10^{-7}	1·3	87
	0·001	5×10^{-7}	0·7	93
	0·005	5×10^{-7}	0·9	91
	0·01	5×10^{-7}	1·6	84
	KBr			
0·1 *M* CH_3COOH, *p*H 2·87	0·0001	5×10^{-7}	1·2	88
	0·001	5×10^{-7}	0·6	94
	0·005	5×10^{-7}	1·5	85
	0·01	5×10^{-7}	2·4	76
	KI			
0·1 *M* CH_3COOH, *p*H 2·87	0·0001	5×10^{-7}	1·1	89
	0·001	5×10^{-7}	0·7	93
	0·005	5×10^{-7}	2·3	77
	0·01	5×10^{-7}	3·3	67

Both the bromide and iodide, like the chloride, when present in very small amounts, accelerated the oxidation of ascorbic acid compared with solutions without halide. Table 4 shows also that the minimum concentration of halide necessary to obtain protection is least with iodide and greatest with chloride.

Influence of p*H*. Kellie & Zilva [1935] observed the beneficial effect of NaCl in preventing the oxidation of ascorbic acid in water. They did not state the *p*H of their solution, but it must have been of the order of *p*H 4·6, for the concentration of ascorbic acid they used was 35 mg./100 ml. Barron *et al.* [1936], who could not confirm Kellie and Zilva's results, used a buffered citrate solution of *p*H 6·0. In view of the findings reported in this paper, these divergent results seem to the writer to be explicable by differences in the *p*H of their solutions.

The influence of *p*H on the protective action of the halide ions has, therefore, been studied in solutions of three different acids, citric, acetic and sulphuric, over a *p*H range varying with the acids from 1·20 to 6·5. No results of such a study are valid unless other factors are also controlled. It is obvious that Cu must be one of these, and, as is pointed out later in the paper, the control of the concentration of hydroxy compounds, especially hydroxy-acids, is important.

Solutions of *p*H varying from 2·30 to 6·50 were prepared, using a mixture of citric acid and sodium citrate, and the oxidation of ascorbic acid, with and without added halide salts, was studied. The concentration of the total citrate, present either as acid or as sodium citrate, was controlled by adding citric acid and sodium citrate in equivalent proportions. Two series of such solutions were

prepared with a total amount of citrate of 0·1 % and 1 %. Cu in all the solutions was maintained constant, in the 1 % citrate solution at $0{\cdot}25 \times 10^{-7}$ and in the 0·1 % solution at approximately $0{\cdot}025 \times 10^{-7}$.

Figs. 6 and 7 illustrate the results obtained; it is clear that the maximum protection given by the chloride is obtained between *p*H values of 2·30–4·00 in both the weaker and the stronger citrate solutions. As the *p*H becomes progressively more alkaline than 4·0 there is an abrupt fall in the protection afforded, to a value approximating to zero in the neighbourhood of *p*H 6·0. The stability

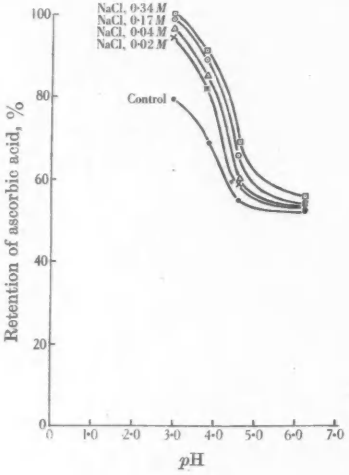

Fig. 6.

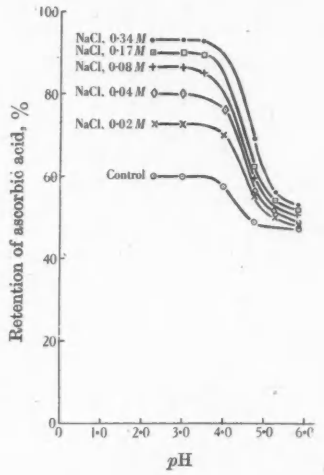

Fig. 7.

Fig. 6. Influence of *p*H on protective action of chloride. Citric buffer (0·1 % total citrate), $Cu = 0{\cdot}025 \times 10^{-7}$, solution heated 30 min. at 100°.

Fig. 7. Influence of *p*H on protective action of chloride. Citric buffer (1·0 % total citrate), $Cu = 0{\cdot}25 \times 10^{-7}$, solution heated 30 min. at 100°.

of ascorbic acid in the control solutions was similarly affected. Results obtained with mixtures of molar acetic acid and sodium acetate solutions, with Cu present in a concentration of 5×10^{-7}, showed the same influence of *p*H on the protective action of the halide ions. Further data, obtained with solutions of 0·1 *N* H_2SO_4, neutralized with NaOH or $NaHCO_3$ to *p*H values ranging from 1·20 to 6·0, support the same conclusion; there was, however, the difference that in solutions more acid than *p*H 2·0, the oxidation of ascorbic acid was considerably reduced, with the result that the protection due to the halide ion was correspondingly decreased. At a *p*H of 1·20, there was no advantage in adding a halide salt; indeed, with the higher concentrations of salt, the action of the halide was deleterious (see Table 5). The explanation of this finding is not clear.

The results indicate that the influence of *p*H on the protective action of the halide ions is significantly affected neither by the nature of the acid radical present, nor by its concentration, nor by the amount of Cu present. It is possible

Table 5

In each instance the solution originally contained 10 mg. ascorbic acid/100 ml.

Solution	Molarity of NaCl added	*p*H	Ascorbic acid after heating 30 min. at 100° mg./100 ml.	% destruction of ascorbic acid
H_2SO_4, 0·1 *N*	—	1·20	9·0	10
	0·04	1·20	9·3	7
	0·08	1·20	9·4	6
	0·17	1·20	9·3	7
	0·25	1·20	8·8	12
	0·50	1·20	7·8	22
H_2SO_4, 0·1 *N* (partly neutralized)	—	1·90	5·4	46
	0·04	1·90	8·1	19
	0·08	1·90	9·0	10
	0·17	1·90	9·2	8
	0·34	1·90	9·4	6
H_2SO_4, 0·1 *N* (partly neutralized)	—	2·30	4·2	58
	0·04	2·30	8·8	12
	0·08	2·30	9·3	7
	0·17	2·30	9·5	5
	0·25	2·30	9·6	4
	0·50	2·30	9·9	1

that other substances not present in these solutions, but present in natural products, may alter the influence of *p*H. This effect of *p*H partly explains why Kellie & Zilva [1935] obtained a positive result with NaCl, and Barron *et al.* [1936] a negative one.

Influence of hydroxy compounds. A further factor of importance is the nature and concentration of other acid anions. If reference be made to Table 1 it is evident that the efficiency with which the added salt prevents the oxidation of ascorbic acid in the different acid solutions, varies considerably. In these preliminary experiments the acids were not used in equivalent concentrations, and—even of more importance—the amount of Cu in different solutions varied. Direct comparison of the efficiency of the halide ion in protecting ascorbic acid from oxidation in these solutions could not therefore be made.

If the concentration of Cu and the *p*H are controlled, and the acids are used in equivalent concentration, direct comparison is possible. A series of experiments has been carried out in which the protective influence of the halide ion has been determined in solutions of the acids at concentrations of 1, 0·1 and 0·01 *M*. Cu was added in all cases to give a concentration of 1 part in 2×10^6, allowance being made for the Cu present as impurity in the acids and other reagents used.

When the natural *p*H of the acid solution together with the ascorbic acid added was lower than *p*H 2·30, it was adjusted with NaOH to 2·30. In the case of the weaker acid solutions, when the *p*H was higher than 2·30 no adjustment was made, since it has already been shown that the protective effect due to halide was constant and at a maximum within a *p*H range of 2·3–4·0.

Experiments have been carried out with citric, tartaric, glycollic, acetic, malic and succinic acids. All the results obtained are in general agreement and indicate that the protective influence of the halide ion varies with the nature and concentration of the acid anion used. With increasing concentration of the hydroxy-acids the protective influence of the halide ion is decreased, whereas acids containing no hydroxyl group have no such influence. For example, NaCl, when added in a concentration of 0·34 *M*, had no protective

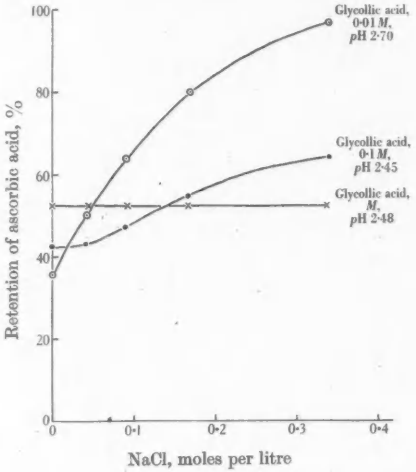
Glycollic acid, 0·01 M, pH 2·70
Glycollic acid, 0·1 M, pH 2·45
Glycollic acid, M, pH 2·48
Retention of ascorbic acid, %
NaCl, moles per litre

Fig. 8. Relation between concentrations of glycollate and chloride in oxidation of ascorbic acid. $Cu = 5 \times 10^{-7}$; 30 min. heating at 100°.

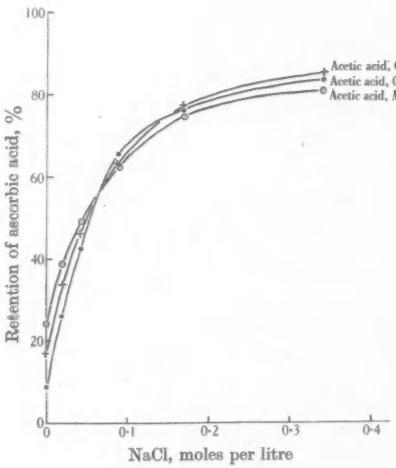

Fig. 9.

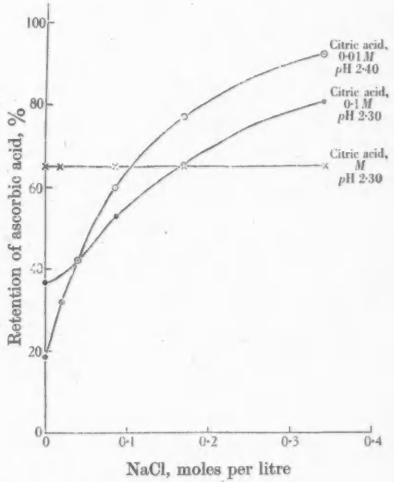

Fig. 10.

Fig. 9. Relation between concentrations of acetate and chloride. $Cu = 5 \times 10^{-7}$; 30 min. heating at 100°.

Fig. 10. Relation between concentrations of citrate and chloride. $Cu = 5 \times 10^{-7}$; 30 min. heating at 100°.

Table 6

In each instance the solution originally contained 10 mg. ascorbic acid/100 ml.

Solution	Molarity of NaCl added	*p*H	Cu present parts/million	Ascorbic acid after heating 30 min. at 100° mg./100 ml.	% destruction of ascorbic acid
Tartaric acid, 0·01 *M*	—	2·30	0·5	3·80	62
	0·02	2·30	0·5	4·00	60
	0·04	2·30	0·5	4·50	55
	0·08	2·30	0·5	5·00	50
	0·17	2·30	0·5	6·80	32
	0·34	2·30	0·5	8·40	16
Tartaric acid, 0·1 *M*	—	2·35	0·5	5·30	47
	0·02	2·35	0·5	4·80	52
	0·04	2·35	0·5	4·90	51
	0·08	2·35	0·5	5·20	48
	0·17	2·35	0·5	6·00	40
	0·34	2·35	0·5	6·30	37
Tartaric acid, 1 *M*	—	2·30	0·5	6·30	37
	0·02	2·30	0·5	6·30	37
	0·04	2·30	0·5	6·10	39
	0·08	2·30	0·5	6·20	38
	0·17	2·30	0·5	5·80	42
	0·34	2·30	0·5	5·60	44
Succinic acid, 0·01 *M*	—	3·07	0·5	2·50	75
	0·04	3·07	0·5	5·40	46
	0·08	3·07	0·5	6·60	36
	0·17	3·07	0·5	7·70	23
Succinic acid, 0·6 *M*	—	2·83	0·5	2·40	76
	0·04	2·83	0·5	4·50	55
	0·08	2·83	0·5	5·60	44
	0·17	2·83	0·5	6·90	31

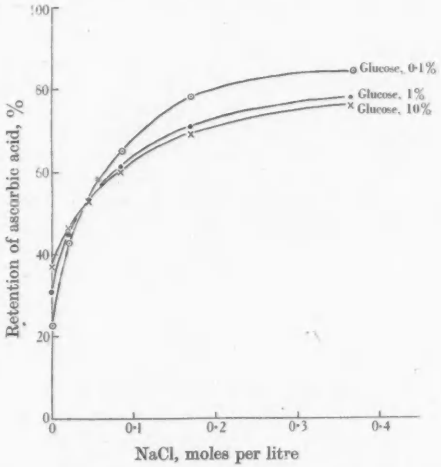

Fig. 11. Influence of glucose on protective action of chloride. Solution: 0·1 *M* acetic acid, *p*H 2·87, Cu = 5×10^{-7}; 30 min. heating at 100°.

influence on the oxidation of ascorbic acid in molar solutions of citric, tartaric, malic or glycollic acids, but in molar solutions of acetic and succinic acids the degree of protection obtained was considerable. Indeed, with the non-hydroxy acids the protective influence of the salt was not markedly different in solutions varying in concentration from 0·01 to 1 *M*. In the weaker solutions (0·01–0·1 *M*) of the hydroxy-acids, the action of the salt was well marked. The results with glycollic, acetic and citric acids are illustrated in Figs. 8, 9 and 10, and with the other acids in Table 6.

In the absence of halide ions the extent of the oxidation of ascorbic acid depends on the nature and concentration of the acid anion. In equivalent concentration the hydroxy-acids depress the oxidation to a greater extent than do acids like acetic, and this effect is most marked in the more concentrated solutions. Indeed, with the non-hydroxy-acids examined, if *p*H is controlled, the oxidation is independent of the concentration of the acid anion. In an experiment with succinic acid at a concentration of 0·01 *M* (*p*H 3·07) and 0·6 *M* (*p*H 2·90) the losses at 100° were 75 and 76 %, respectively.

Other hydroxy compounds behaved like the hydroxy-acids. Fig. 11 shows the results of an experiment in which the effect of increasing amounts of glucose on the protective action of the halide ion was determined. Here, as with the hydroxy-acids, as the concentration of the sugar was increased, so the degree of protection due to the halide ions was reduced, though not to the same marked extent as with the hydroxy-acids. The same effect was seen with ethyl alcohol.

Part II. Influence of halide ions on the oxidation at 20°

The results outlined in Part I of this paper have shown that the stability of ascorbic acid in solution at 100° depends on the relative amounts of Cu and halide ions present, and that the action of these ions is modified by change in *p*H, and by the presence of hydroxy compounds. To determine the influence of temperature on this system, the oxidation of ascorbic acid has also been studied at a lower temperature, viz. 20°. The catalytic influence of Cu on the oxidation is increased by a rise of temperature, Barron *et al.* [1936] reporting a temperature coefficient of 1·65 for the reaction. The experiments to be described below fully support these conclusions, and in addition show how the action of the halide ion is influenced by temperature.

At 100° the stability of ascorbic acid was tested by determining its loss over a given period of time (30 min.). At 20° the solubility of O_2 in the solutions used was sufficient to allow the reaction ascorbic acid → dehydroascorbic acid to proceed to completion. This enabled the rate of oxidation to be readily followed. The reaction was studied at 20° in solutions of 0·1 *M* acetic acid at *p*H 2·87, or in 1 % citric acid at *p*H 2·35, in the presence of varying amounts of Cu. The influence of chloride, added as NaCl, in concentrations varying from 0·02 to 1·6 *M* was examined. Two experiments in this series, typical of others, are illustrated in Figs. 12 and 13. The general results show that the influence of the halide ion at 20°, as at the higher temperatures, depends on its concentration. With the lower concentrations there is a marked increase in the rate at which the ascorbic acid is oxidized, compared with control solutions without halide, and not until a considerable amount of halide is present is the oxidation reduced below the rate for the control. Qualitatively, the results are similar to those obtained at 100°. At the lower temperature, however, chloride must be present in an amount considerably in excess of that necessary under corre-

sponding conditions at 100° in order to suppress the oxidation to the same extent. These results are the more remarkable when it is remembered that the catalytic activity of Cu is much greater at the higher temperature.

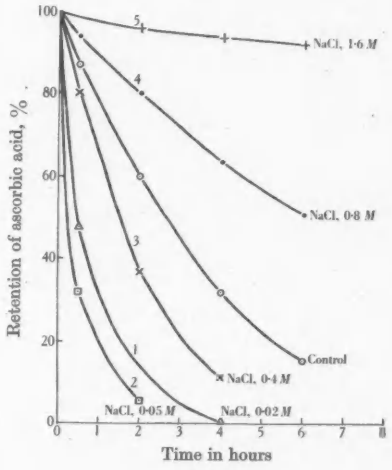

Fig. 12.

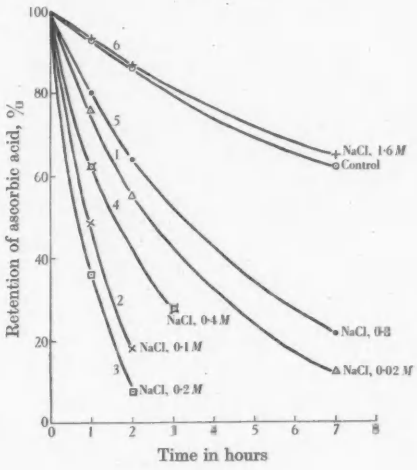

Fig. 13.

Fig. 12. Rate of oxidation of ascorbic acid at 20° as influenced by varying concentrations of NaCl. Solution: 0·1 *M* acetic acid, *p*H 2·87, $Cu = 25 \times 10^{-7}$. Numbers on curve refer to order of concentration of NaCl used.

Fig. 13. Rate of oxidation of ascorbic acid at 20° as influenced by NaCl in presence of a hydroxy-acid. Solution: 1% citric acid, *p*H 2·35, $Cu = 25 \times 10^{-7}$. Numbers on curve refer to order of concentration of NaCl used.

The graphs in Figs. 14 and 15 show more clearly the relationship between the concentrations of Cu and chloride, and the stability of the ascorbic acid. The ordinates represent the percentage of ascorbic acid retained during a period of 30 min. at 20°. These values were obtained either by direct experimental observation or by intrapolation from curves such as those shown in Figs. 12 and 13. The results are thus directly comparable with those in the similar experiments carried out at 100° and illustrated in Figs. 3 and 4. For ease of comparison the former graphs have been replotted on the same scale and are shown as dotted lines in Figs. 14 and 15.

At the lower temperature the deleterious action of the chloride, in the weaker concentrations, is more pronounced, both in the magnitude of the destruction brought about and also in the range of concentration throughout which its influence extends. It should, however, be noted that the amount of NaCl necessary to suppress completely the oxidation in the presence of a given concentration of Cu is not appreciably modified by change of temperature. As at the higher temperature, the extent to which the chloride adversely affects the stability of ascorbic acid depends on the amount of Cu present. For example, in 0·1 *M* acetic acid at 20°, with a concentration of Cu of $0{\cdot}12 \times 10^{-7}$, the maximum extra loss over that in the control solution is 3·5%, and no protection is obtained until the chloride is present in a concentration of 0·1 *M*,

whereas in the same solution with Cu at 25×10^{-7}, the maximum extra loss is 60%, and protection is only obtained when the halide ion is present in a concentration greater than $0{\cdot}55\,M$.

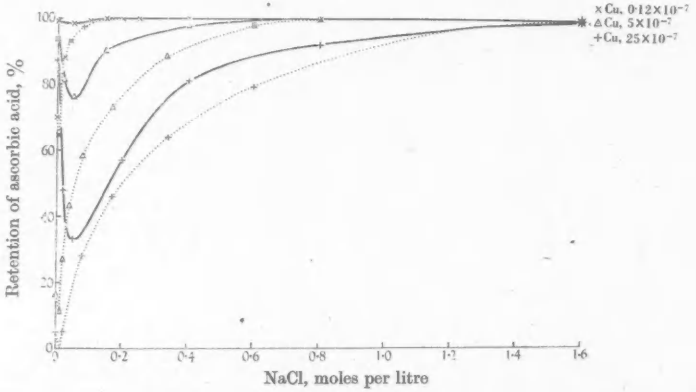

Fig. 14. Comparison of effects of varying concentrations of chloride and Cu, at 20° and 100°. Solution: $0{\cdot}1\,M$ acetic acid, *p*H 2·87. Oxidation period = 30 min. —— Solution at 20°, ····· solution at 100°.

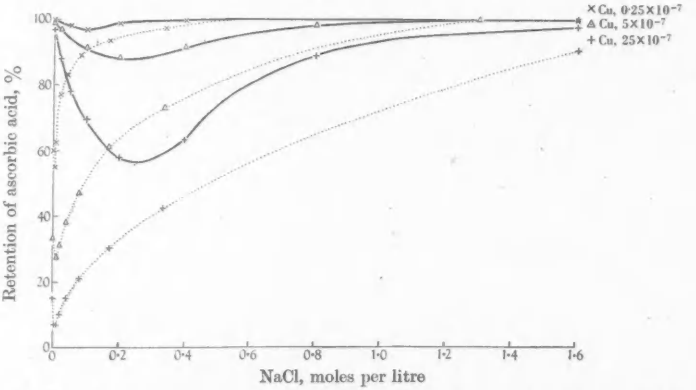

Fig. 15. Influence of varying concentrations of chloride and Cu, at 20° and 100°, in solution of a hydroxy-acid. Solution: 1·0% citric acid, *p*H 2·35. Oxidation period = 30 min. —— Solution at 20°, ····· solution at 100°.

Efficiencies of the chloride, bromide and iodide at 20°. Quantitatively the same results have been obtained with the two other halide salts, bromide and iodide. Fig. 16 shows the relative influence of the three halides on the oxidation during a period of 2 hr. at 20° in a solution of acetic acid ($0{\cdot}1\,M$). The efficiencies of the ions in suppressing the oxidation of ascorbic acid are chloride < bromide < iodide.

If we compare the results illustrated in Fig. 16 and the corresponding results at 100° (Fig. 5), it is evident that in contrast with chloride and bromide the protective influence of the iodide is not significantly altered by temperature.

A summary of these data is given in Table 7; this shows that an increase of temperature from 20 to 100° decreases by 1/20 and 1/15 respectively, the amount of chloride and bromide that it is necessary to add to depress the oxidation of ascorbic acid below that in a control solution without halide. The concentration of the iodide remains unaltered.

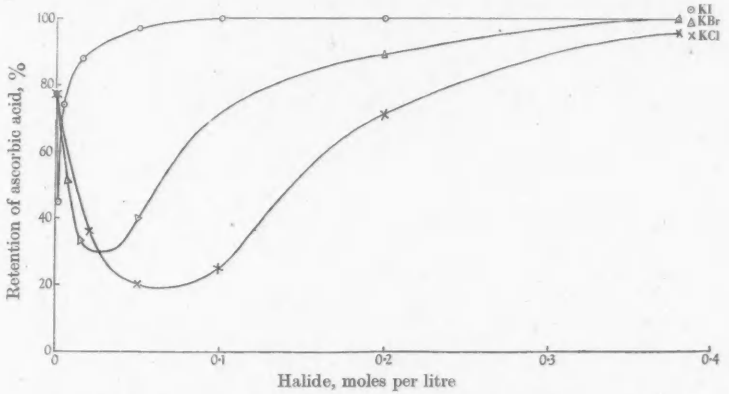

Fig. 16. Relative effects of different halides on oxidation of ascorbic acid at 20°. Solution: 0·1 *M* acetic acid, *p*H 2·87, Cu = 5×10^{-7}, oxidation period = 2 hr.

The highest concentrations shown in the last column represent the smallest amount of each halide salt which it was necessary to add to suppress entirely the oxidation of ascorbic acid in the acetic acid solution. For each salt these concentrations, it will be noted, are not significantly affected by change in temperature.

Table 7

Solution	Temperature	Range of concentration of the halide ion throughout which the stability of ascorbic acid is decreased	Concentrations of halide ion throughout which the stability is increased
Acetic acid, 0·1 *M*, with Cu present in a concentration of 5×10^{-7}	20°	KCl 0–0·23 *M*	0·23 –0·52 *M*
	20°	KBr 0–0·12 *M*	0·12 –0·38 *M*
	20°	KI 0–0·005 *M*	0·005–0·11 *M*
	100°	KCl 0–0·011 *M*	0·011–0·50 *M*
	100°	KBr 0–0·008 *M*	0·008–0·32 *M*
	100°	KI 0–0·005 *M*	0·005–0·12 *M*

Influence of O_2. The influence of the concentration of O_2 upon the stability of ascorbic acid in the presence of chloride has been determined at 20°. The rate of oxidation of the acid was measured in a solution of 0·1 *M* acetic acid, with Cu present in a concentration of 5×10^{-7}. Separate solutions had previously been brought into equilibrium with either O_2, air or a gas mixture containing 5 % O_2 and 95 % N_2 (Fig. 17), and this equilibrium was maintained throughout the experiment by the continuous bubbling of the gases through their respective solutions. The results are summarized in Fig. 18 and show that an increase in the concentration of O_2 enhances the destructive influence of the chloride. It is worth noting that the oxidation of the acid in the control solu-

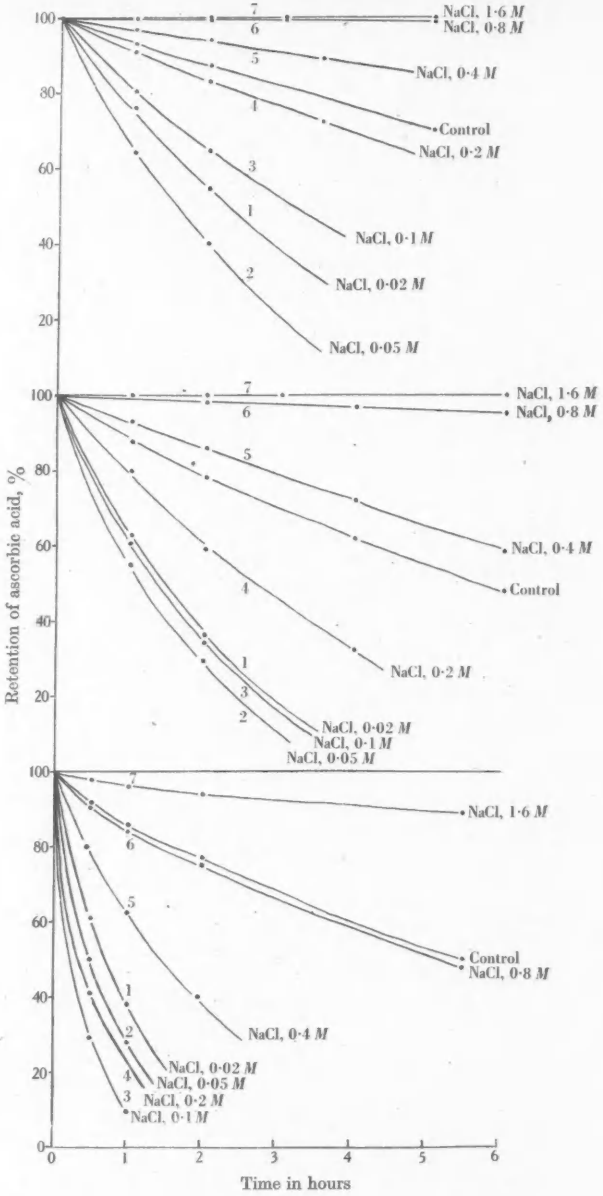

Fig. 17. Rate of oxidation of ascorbic acid at 20° as influenced by O_2 and concentration of chloride. Solution: 0·1 *M* acetic acid, *p*H 2·87, Cu = 5×10^{-7}. Top panel = 5% O_2 + 95% N_2. Middle panel = air. Bottom panel = 100% O_2. Numbers on curves refer to order of concentration of NaCl used.

tions was not increased when air was replaced by pure O_2. A reduction in the rate of oxidation occurred, however, when air was replaced by the gas mixture of 5 % O_2 and 95 % N_2.

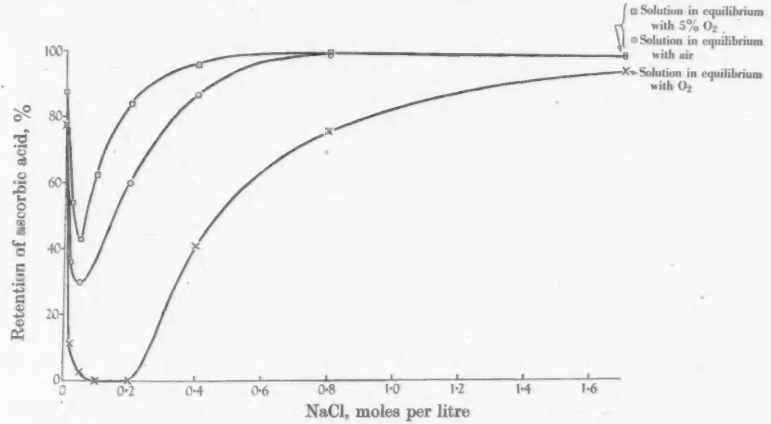

Fig. 18. Action of chloride at 20° as influenced by amount of available O_2. Solution: 0·1 *M* acetic acid, *p*H 2·87, Cu = 5×10^{-7}. Oxidation period = 2 hr.

Discussion

The evidence presented in this paper shows that the oxidation of ascorbic acid catalysed by Cu is influenced to a large extent by the addition of the halide salts, chloride, bromide and iodide. At a temperature of 100° chloride and bromide were relatively more effective in suppressing the oxidation than at lower temperatures, chiefly because the range of concentrations throughout which their deleterious action was observed was decreased. In practice this means that a greater concentration of the halide salt is necessary to suppress the oxidation of the acid at ordinary temperatures. In contrast, the action of iodide is not significantly altered by change in temperature. The action of the halides at all temperatures is correlated with the amount of Cu present, for the extent both of their destructive and protective influences varies with the concentration of the Cu.

The complete removal of Cu from a solution renders ascorbic acid stable to O_2 and unaffected by the presence or absence of halide ions. Very small amounts of Cu are sufficient at a temperature of 100° to catalyse the oxidation of the acid; in the experiments reported here, Cu had to be reduced to approximately $0{\cdot}015 \times 10^{-7}$ before the oxidation of the acid was virtually suppressed.

It seems probable that the inhibitory action of the halide ions is due to the formation of a complex ion between them and Cu. An explanation of the destructive influence of the halide ion is far from clear. The fact that the magnitude of this effect is connected with the amount of Cu present indicates that Cu participates in these reactions. A plausible explanation is that the halide ion forms a hydroxy-acid complex, and the O_2 so activated is more readily available with Cu for the catalytic oxidation of ascorbic acid. When sufficient halide has been added, the copper-halide complex is formed and the oxidation

thereby suppressed. Admittedly no evidence in support of this explanation has been obtained, apart from the fact that the destructive influence is more marked as the O_2 in the solution is increased or as the temperature is lowered—both conditions in which the formation of such a hypothetical oxy-halide ion should be favoured.

The precise mechanism of the action of the hydroxy-acids and other hydroxy compounds in decreasing the effectiveness of the halide salts is also not clear. It seems probable that they act in the same manner as these salts, by forming complexes with Cu. If this is true, they must compete with the halide ions for Cu, for the higher the concentration of the hydroxy compound, the greater the concentration of salt needed to obtain the halide effect. Moreover, in solutions like molar citric acid, in which there is no protective influence of added salt up to concentrations of 0·34 *M*, the percentage loss of ascorbic acid is of the order of 36, but in solutions of 0·01 *M* citric acid with the same concentration of salt, the percentage destruction of ascorbic acid has been reduced to 6. Any hypothetical complex formed between the citrate ion and Cu must, therefore, prevent the formation of the complex between halide and Cu. This, however, presupposes 100 % complex formation between Cu and citrate, and yet oxidation of ascorbic acid is not under these conditions reduced to zero. The implications of this argument suggest that the complex formed between Cu and hydroxy compound still retains some catalytic activity.

Whatever the real explanation, from the practical point of view it is clear that a greater retention of the vitamin can be obtained in a solution of a weak hydroxy acid plus salt than in a solution of a more concentrated hydroxy acid without salt at 100°. In a continuation of this work it is hoped to throw more light on these points.

The degree of protection of ascorbic acid which can be secured by adding halide salts to any foodstuff or processed extract will depend on the concentration in which they are added, on the temperature, on the *p*H, on the content of Cu and on the nature and concentration of other substances such as hydroxy-acids and sugars. It is probably true that in a complex system like a foodstuff, other factors not recognized here may influence the action of these salts and speculation is therefore unprofitable. It is certainly true, however, that these halide effects would appear to be more generally applicable to fresh fruits and processed fruit products, than to vegetables which have a natural *p*H of 6·0–7·0 and are not generally processed in acid media.

The degree to which salt can be used with advantage in foods to preserve vitamin C will depend not only on the factors enumerated above but on the amount of salt it is possible to add without detracting from taste and flavour. This limit will vary with different foods, but concentrations of salt of from 1 to 2 % can be used in cooking vegetables, and concentrations up to 0·5 % can be added to most fruits, without affecting palatability.

Summary

1. The oxidation of ascorbic acid was studied under standardized conditions in various acid solutions heated to 100° for 30 min.

2. Ascorbic acid was not oxidized at 100° if Cu was reduced to 1 part in 650×10^6 of solution.

3. The oxidation was greatly reduced if chloride, bromide or iodide salts were added in sufficient amount, the extent of the reduction being proportional to the concentration of the halide present. In weaker concentrations, however,

the halides had a slight destructive effect. Both the destructive and protective influences of the salts were correlated with the amount of Cu present.

4. The action of the halide salts was independent of the nature of the cation, though slightly influenced by the valency of the salt.

5. The relative efficiencies of the three halides in stabilizing ascorbic acid were of the order, iodide > bromide > chloride. Fluoride had no protective action.

6. The protective influence of the halides was operative over a *p*H range of 2·0–6·0, with a maximum effect at *p*H 2·3–4·0, but diminished rapidly between 4·0 and 6·0, or below 2·0. At *p*H 1·20 a high concentration of halide was deleterious.

7. Hydroxy-acids antagonized the protective action of the halide salts.

8. The influence of the halides on the rate of oxidation of ascorbic acid at 20° corresponded qualitatively with their influence at 100°. Quantitatively, however, the destructive effects of chloride and bromide at lower concentrations were markedly increased, but that of the iodide not significantly affected by the alteration of temperature.

9. An increase in the concentration of O_2 enhanced the destructive influence of the halides at 20°.

I wish to thank Drs C. S. Hanes and F. H. Isherwood for valuable suggestions, and Dr L. J. Harris for his helpful criticism and advice; to Mr L. D. Ward I wish to express my thanks for his technical assistance.

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150. EFFECT OF DIETS RICH IN PROTEIN UPON RATS DEPRIVED OF VITAMIN B_1

By GOUTOM G. BANERJI

From the Nutritional Laboratory, University of Cambridge and Medical Research Council

(*Received 7 November 1941*)

In a previous communication [Banerji, 1940] it was noted that the ingestion of a diet rich in fat caused a diminution in the excretion of bisulphite-binding substances in the urine of rats deficient in vitamin B_1. It was hence thought worth while to investigate the effect of a high-protein diet on the excretion of these compounds by the deficient rats.

Experimental

The bisulphite-binding substances in the urine were measured by the modified method used in the last experiment of the previous communication [Banerji, 1940].

The electrocardiogram was used for taking heart-rates in the way described by Drury & Harris [1930] and Birch & Harris [1934].

The rats used in these experiments were young males weighing between 60 and 90 g.

The compositions of the diets used are given below (Table 1).

Table 1

	High protein diets (A)	High protein diets (B)	Standard basal diet
Sucrose	0	0	60
Lard	15	0	0
Arachis oil	0	15	15
Light white casein	80	80	20
Salt mixture	5	5	5
Marmite ('laboratory' brand), autoclaved at *p*H 8–9, 1½ atmospheres pressure for 1 hr.	12	12	12
Cod-liver oil	2 drops daily		2 drops daily

Each rat was kept isolated throughout the experimental period to lessen complications from coprophagy, refection etc.

Results

(1) *Effect of high-protein diet in the absence of vitamin B_1*

As is seen from Fig. 1, the rats on a diet deficient in vitamin B_1 but containing the high proportion of protein, although they excreted a large amount of bisulphite-binding substances (B.B.S.), neither died nor developed bradycardia. Similar results were obtained in a duplicate experiment.

(2) *Comparison of the excretion of* B.B.S. *with and without high protein, and with graded supplements of aneurin*

Diet B of Table 1 was used in this experiment with graded doses (0, 2 and 10 μg. daily) of vitamin B_1. The vitamin was injected intraperitoneally. It had

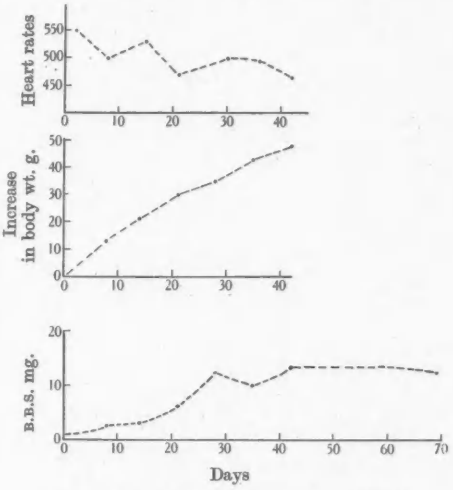

Fig. 1. High-protein diet, fat as arachis oil (average of 3 rats).

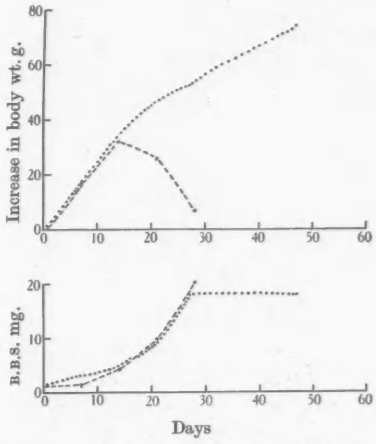

Fig. 2. ··· High protein diet +no vitamin B_1.
--- Ordinary basal diet + no vitamin B_1.

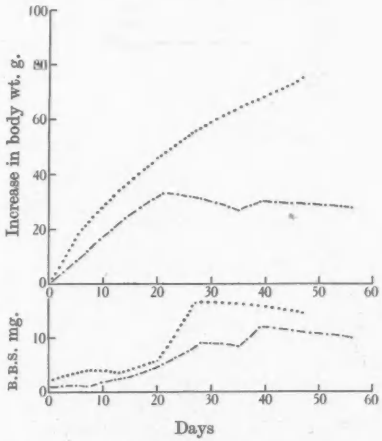

Fig. 3. ··· High protein + 2 μg. vitamin B_1.
—·— Ordinary basal diet + 2 μg. vitamin B_1.

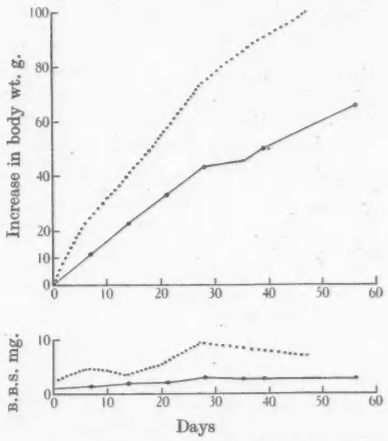

Fig. 4. ··· High protein diet + 10 μg. vitamin B_1.
—— Ordinary basal diet + 10 μg. vitamin B_1.

the usual accelerating effect on growth, and the level of the excretion of B.B.S. varied inversely with the amount of the vitamin injected (Figs. 2–4). The rate

of growth was higher on the high protein diet for each level of intake of the vitamin.

The group of rats on the standard basal diet started losing weight after about a fortnight. The animals then developed bradycardia and died in 4 weeks from the beginning of the experiment. The group on the vitamin B_1-free high-protein diet, on the other hand, went on gaining weight week by week (Fig. 2). The excretions of B.B.S. by the rats on the two diets when having no vitamin B_1 were found to be very much the same.

A comparison of the two groups of rats having 2μg. of vitamin B_1 per rat per day (Fig. 3) shows that the group on the standard basal diet maintained its growth after attaining a given weight, whereas there was a gradual increase of growth in the group having the high protein. There was, however, no significant difference in their excretion of B.B.S. (Fig. 3).

Daily supplements of 10μg. of vitamin B_1 per rat per day (Fig. 4) caused a continuous gain in weight in both the groups. The rate of growth, however, was greater in the group on the high-protein diet. The excretion of B.B.S. was very low and was maintained at approximately the same level in both groups.

Examination of casein for vitamin B_1. In order to determine whether the action of the casein in preventing loss of weight and bradycardia was due to its possible contamination with vitamin B_1, the casein was analysed for the vitamin according to the method of Wang & Harris [1939] and Harris & Wang [1941].

50 g. of the light-white casein were heated on a water-bath with about 500 ml. of distilled water in a beaker until dissolved. The reaction was then adjusted to *p*H 2 and the beaker was kept in an incubator at 40° overnight with the addition of 1 g. of pepsin. The reaction was next adjusted to *p*H 4 and the beaker was left in the incubator at 40° overnight with the addition of 0·5 g. takadiastase. The *p*H was then adjusted to 4·7 to precipitate the casein, and the mixture was centrifuged. The clear centrifugate was then shaken with 1 g. of fuller's earth at *p*H 4 for the adsorption of the vitamin. It was next filtered and dried. The weight of the dried fuller's earth was found to be 1·66 g. 0·05 g. of this fuller's earth was then examined for its vitamin B_1 content by the thiochrome method, according to the procedure of Wang & Harris [1939], and was found to contain 0·18μg. of vitamin B_1.

Hence 1 g. of light-white casein contains 0·119μg. of vitamin B_1.

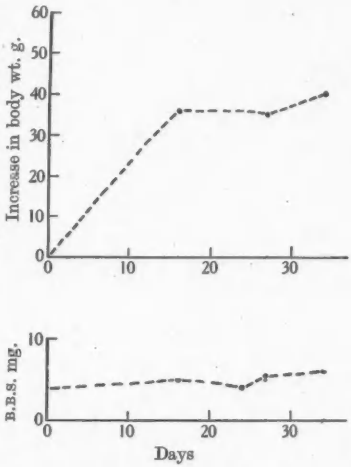

Fig. 5. High-protein diet, with extracted casein (average of 3 rats).

Though the amount of vitamin B_1 present in the light-white casein is thus too small to explain the significant difference observed in the above experiments, the next experiment was nevertheless carried out with light-white casein which had been extracted by the acid-alcohol method of Guha & Drummond [1929]. This extracted casein was tested in the above way and found to be almost free from vitamin B_1.

The vitamin B_1-deficient high-protein diet containing this extracted casein permitted a gradual increase of growth and there was some increased excretion

of bisulphite-binding substances (Fig. 5). In this experiment, however, the B.B.S. excretion was not very high: this was possibly due to the fact that the rats only consumed small quantities of the diet.

Discussion

The principal conclusion which stands out from these experiments is that protein, in the form of light-white casein, spares vitamin B_1. Further proofs have been secured, based on (*a*) the vitamin content of the tissues and (*b*) the catatorulin effect of the rats' kidney on vitamin B_1-deficient high-protein, high-fat and standard-basal diets. This work has been done in collaboration with Dr J. Yudkin, and an account of it is in the press [Banerji & Yudkin, 1941].

Investigations are in progress to determine whether proteins other than the light-white casein spare vitamin B_1. It is also intended to extend these experiments by using individual amino-acids.

SUMMARY

1. The presence of a large amount of protein in the diet prevented the bradycardia and loss in weight usually associated with deficiency of vitamin B_1. It failed however to prevent the increased excretion of bisulphite-binding substances. In this last respect, protein differs from fat, which has previously been shown to relieve this as well as the other effects of deficiency.

2. The vitamin-sparing action of the protein was demonstrated in further experiments in which the growth rates on various graded levels of vitamin B_1 were found to be better in each case with excess of protein than without it. There was a similar effect on the urinary excretion of B.B.S.

The author wishes to take this opportunity to express his gratitude to Dr L. J. Harris for his continual interest and helpful criticisms.

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151. A NEW FORM OF CHROMATOGRAM EMPLOYING TWO LIQUID PHASES

1. A THEORY OF CHROMATOGRAPHY

2. APPLICATION TO THE MICRO-DETERMINATION OF THE HIGHER MONOAMINO-ACIDS IN PROTEINS

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(*Received 19 November 1941*)

Introduction

In most forms of counter-current extraction column the very small drop required for the rapid attainment of equilibrium, and hence for high efficiencies, cannot be used owing to the difficulty of preventing it moving in the wrong direction. In the case of a solid, however, for any reasonable size of particle a filter will prevent movement in any undesired direction. Consideration of such facts led us to try absorbing water in silica gel etc., and then using the water-saturated solid as one phase of a chromatogram, the other being some fluid immiscible with water, the silica acting merely as mechanical support. Separations in a chromatogram of this type thus depend upon differences in the partition between two liquid phases of the substances to be separated, and not, as in all previously described chromatograms, on differences in adsorption between liquid and solid phases.

The difficulties of using chromatograms are very greatly lessened when the substances to be separated are coloured, or if colourless can be made visible. Various methods have been used for this [cf. Zechmeister & Cholnoky, 1936; Cook, 1941], though none of these was suitable for our problems. As the substances which we desired to separate were acids, and water was one of our phases, we were able to obtain visual evidence of the presence of any of these acids by adding a suitable indicator to the water with which the gel was saturated.

In the present paper we present an approximate theory of chromatographic separations, and describe an application of the new chromatogram to the micro-determination of the higher monoamino-acids in protein hydrolysates. This method is based on the partition of acetamino-acids between chloroform and water phases, and supersedes the macro-method described by us [Martin & Synge, 1941, 1], being rapid and economical both of materials and of apparatus.

Work is in progress, using ethyl acetate as the less polar phase in the chromatogram, on the separation of the acetyl derivatives of most of the other naturally occurring amino-acids, and the method promises also to be of use in analogous separations of simple peptides.

We wish to stress, however, that the possible field of usefulness of the new chromatogram is by no means confined to protein chemistry. By employing suitable phase pairs, many other substances should be separable. Where water is suitable as one of the phases, an indicator may be used to render visible the separation of organic acids or bases. Even where this is not possible, as with

neutral substances, the theory given below will enable 'cuts' of known partition coefficient to be taken. In the ordinary adsorption chromatogram optically active adsorbents have been employed for optical resolutions [cf. Henderson & Rule, 1937; Karagunis & Coumoulos, 1938], and resolution of racemic substances may be expected in the new chromatogram when either phase is optically active [cf. Bailey & Hass, 1941].

The mobile phase need not be a liquid but may be a vapour. We show below that the efficiency of contact between the phases (theoretical plates per unit length of column) is far greater in the chromatogram than in ordinary distillation or extraction columns. Very refined separations of volatile substances should therefore be possible in a column in which permanent gas is made to flow over gel impregnated with a non-volatile solvent in which the substances to be separated approximately obey Raoult's law. When differences of volatility are too small to permit of ready separation by these means, advantage may be taken in some cases of deviation from Raoult's law, as in azeotropic distillation.

Possibly the method may also be found to be of use in the separation of isotopes, e.g. of nitrogen isotopes, by passing ammonia gas over gel impregnated with ammonium sulphate solution [cf. Urey *et al.* 1937].

1. A THEORY OF CHROMATOGRAPHY

The chromatogram has for a long time been realized to be closely analogous in its mode of operation to distillation and extraction fractionating columns. No one, however, seems to have attempted to work out in detail the theory of the chromatogram using the concepts developed for distillation. This we attempt to do here, and thus to give a picture of the concentration of solute at any time and place in the column, and of the way in which the resolution depends upon the length of the column.

The behaviour of a column consisting of a number of 'theoretical plates', within each of which perfect equilibrium between the two phases occurs, can be described with great simplicity. Peters [1922] showed that the continuous or packed type of distillation column (in which equilibrium is not established at any point) could be divided up into a number of layers each of which was equivalent to one theoretical plate, and the height of such a layer was called the H.E.T.P. or 'height equivalent to one theoretical plate'. For the present purpose the H.E.T.P. is defined as the thickness of the layer such that the solution issuing from it is in equilibrium with the mean concentration of solute in the non-mobile phase throughout the layer. It can be shown from diffusion arguments that the H.E.T.P. is a constant through a given column except when the ratio of the concentrations of the solution entering and leaving the plate differs greatly from unity [cf. Sherwood, 1937]. It may be taken as constant for the chromatogram without serious error.

For the equations to be manageable certain simplifying assumptions must be made, viz. that the diffusion of solute from one 'plate' to another must be negligible, and that at equilibrium the distribution ratio of one solute between the two phases must be independent both of the absolute value of its concentration and of the presence of other solutes.

We consider here a chromatogram of the new type, having two liquid phases. The extension of the theory to the usual adsorption chromatogram is obvious

when the adsorption isotherm is a linear function of the concentration of solute in the liquid phase.

Consider a chromatogram of many 'plates':

Let h = the H.E.T.P.,

A = the area of cross-section of the column,

A_S = the area of cross-section of the non-mobile phase,

A_L = the area of cross-section of the mobile phase,

A_I = the area of cross-section of inert solid ($A_S+A_L+A_I=A$),

v = the volume of solvent used in development of the chromatogram,

α = the partition coefficient, i.e.

$$\frac{\text{g. solute per ml. of non-mobile phase}}{\text{g. solute per ml. of mobile phase}} \text{ at equilibrium,}$$

$V = h(A_L+\alpha A_S)$,

$$R = \frac{\text{movement of position of maximum concentration of solute}}{\text{simultaneous movement of surface of developing fluid in empty part of tube above chromatogram column}},$$

r = serial number of 'plate', measured from top of column downwards,

Q_r = total quantity of solute in plate r.

Consider the case where unit mass of a single solute is put into the first plate, and is then followed by pure solvent. We can draw up a table showing the quantity of solute in each plate after successive infinitesimal volumes of mobile phase δv have passed:

Vol. of solvent passed ($x\delta v$)	Serial number of plate				
	$r=1$	2	3	4	5
0	1	0	0	0	0
1	$(1-\delta v/V)$	$\delta v/V$	0	0	0
2	$(1-\delta v/V)^2$	$2(1-\delta v/V)\,\delta v/V$	$(\delta v/V)^2$	0	0
3	$(1-\delta v/V)^3$	$3(1-\delta v/V)^2(\delta v/V)$	$3(1-\delta v/V)(\delta v/V)^2$	$(\delta v/V)^3$	0
4	$(1-\delta v/V)^4$	$4(1-\delta v/V)^3(\delta v/V)$	$6(1-\delta v/V)^2(\delta v/V)^2$	$4(1-\delta v/V)(\delta v/V)^3$	$(\delta v/V)^4$

We see that the quantity in each plate is a term of the binomial expansion $((1-\delta v/V)+\delta v/V)^n$ so that when n successive volumes of solvent δv have passed,

$$Q_{r+1}=\frac{n!\,(1-\delta v/V)^{n-r}(\delta v/V)^r}{r!\,(n-r)!}.$$

Now when n is large, this becomes

$$Q_{r+1}=\frac{1}{r!}(n\delta v/V)^r e^{-n\delta v/V}.$$

But $n\delta v$ is the volume of solvent that has been used to develop the chromatogram. Putting $n\delta v=v$,

$$Q_{r+1}=\frac{1}{r!}(v/V)^r e^{-v/V},$$

which, by Stirling's approximation, becomes

$$Q_{r+1}=\frac{1}{\sqrt{(2\pi r)}}(v/rV)^r e^{r-v/V}, \qquad \text{.....(1)}$$

when r is large (>10).

Movement of band

Now Q_{r+1} is a maximum and equals $\frac{1}{\sqrt{(2\pi r)}}$ when $v/rV=1$, so that the position of maximum concentration has moved a distance hv/V directly proportional to the volume of liquid v which has flowed through. In terms of the movement of the surface of the liquid standing above the solid in the tube, the relative rate of movement R is given by the expression

$$R=\frac{vh/V}{v/A}, \text{ i.e. } \frac{\text{movement of band}}{\text{movement of surface}}$$

$$=\frac{Ah}{V}$$

$$=\frac{A}{A_L+\alpha A_S}=\frac{A_L+A_S+A_I}{A_L+\alpha A_S},$$

or

$$\alpha=\frac{A}{RA_S}-\frac{A_L}{A_S}. \qquad \text{.....(2)}$$

Separations ideally obtainable

If we plot the concentration of solute in plate $r+1$ against v/V using equation (1) we obtain the curve shown in Fig. 1. When r is infinite this curve

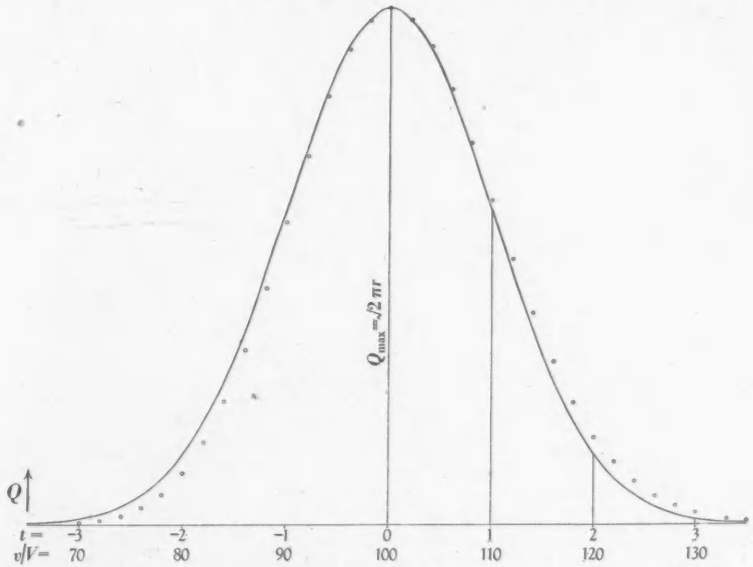

Fig. 1. The points represent the relation between Q and v/V for $r=100$. Cf. equation (1). The full line is the normal curve of error with abscissa t, i.e. $Q/Q_{max.}=\exp[-\frac{1}{2}t^2]$.

becomes the normal curve of error. When $r>100$ the ordinates of the curve can be expressed in terms of the normal curve of error by

$$\frac{v}{V}=r+t\sqrt{r+\frac{t^2}{3}}.$$

Similarly, the area under the curve can be expressed by

$$\frac{v}{V}=r+t\sqrt{r+\frac{t^2}{4}},$$

where t is the abscissa of the error curve.

Characteristics of the curve for various values of t are shown below:

t	$Q/Q_{max.}$	Area under tail of curve as % of whole area under curve
1	0·605	15·9
2	0·135	2·27
3	0·011	0·13

Now if two solutes be present with partition coefficients α and β, then practically complete separation will be obtained when $t=3$; then

$$\frac{A_L+\alpha A_S}{A_L+\beta A_S}=\frac{r-3\sqrt{r+2{\cdot}25}}{r+3\sqrt{r+2{\cdot}25}},$$

since only $<0{\cdot}2\,\%$ of the substance whose partition coefficient is β has passed the $(r+1)$th plate and $>99{\cdot}8\,\%$ of the other substance whose partition coefficient is α has passed it. Fig. 2 shows graphically the relation between $\frac{A_L+\alpha A_S}{A_L+\beta A_S}$ and r for various values of t, i.e. for various degrees of separation.

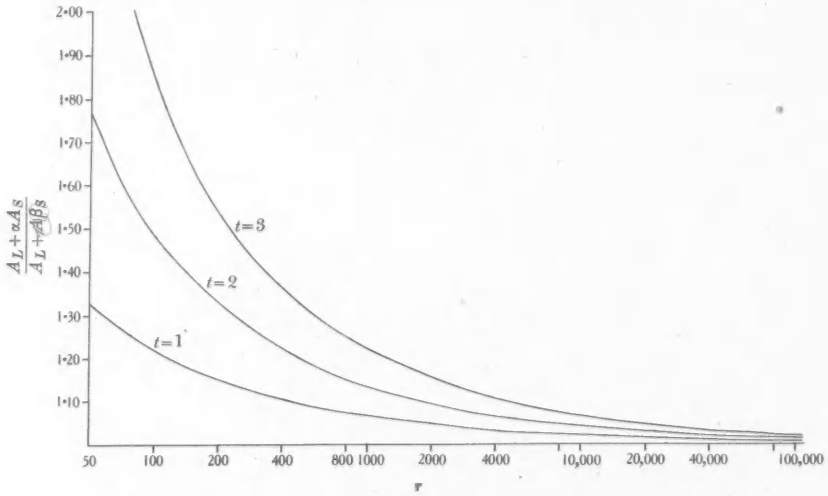

Fig. 2. Relation between difference in partition coefficient, number of plates (r) and degree of separation (t).

It follows from the assumption that the partition coefficient is a constant that there will be no interaction between various chromatograms occurring simultaneously in the same tube. Hence if, as in the practical case, the whole of the solute was not initially in the first plate, but distributed over a number, then the concentration at any plate and time will be the sum of the concentrations for the relevant number of chromatograms started successively. Thus the region of maximum concentration will be broadened, and the total width

of the band will be greater than that shown in Fig. 1 approximately by the initial width of the band before development by solvent was begun. The number of plates required for resolution will be correspondingly increased.

Factors influencing the H.E.T.P.

The height equivalent to a theoretical plate depends upon the factors controlling diffusion and upon the rate of flow of the liquid. There is an optimum rate of flow in any given case, since diffusion from plate to plate becomes relatively more important the slower the flow of liquid and tends of course always to increase the H.E.T.P. Apart from this, the H.E.T.P. is proportional to the rate of flow of liquid and to the square of the particle diameter. Thus the smallest H.E.T.P. should be obtainable by using very small particles and a high pressure difference across the length of the column. The H.E.T.P. depends also on the diffusibility of the solute in the solvent employed, and in the case of large molecules, such as proteins, this will result in serious decrease in efficiency as compared with solutes of molecular weights of the order of hundreds.

Practical limitations of theory

The separations obtainable in practice are less than the theory predicts for two principal reasons. First, the partition coefficient is seldom a constant, usually decreasing as the solution becomes stronger. This results in the front of the band becoming steeper, and the back flatter and, more importantly, in the band becoming wider, since the concentrated part moves faster than the dilute part. This effect can sometimes be diminished by working with initially dilute solutions.

Interaction between two solutes often, however, leads to an increase in separation over the theoretical, the more strongly absorbed solute 'eluting' the less strongly absorbed, and thus tending to cause a sharp boundary between the two, reminiscent of the behaviour of ions in the moving boundary in transport number determinations.

The other great source of loss of efficiency lies in lack of uniformity of flow through the column. This lack of uniformity often prevents good separations being realized even though the solutes be separated in the column itself, as the cut cannot follow the required surface. In striving for conditions for uniformity of flow, the high pressure and small particle size desirable for smallest H.E.T.P. have to be abandoned.

Discussion

Apparently the only attempt at any theory of the chromatogram so far published is that of Wilson [1940]. He neglects, in his treatment, all diffusion and lack of equilibrium, i.e. in our nomenclature, assumes that h is zero. He attempts a general solution for the case that the partition coefficient (adsorption isotherm) is not constant (linear).

Unfortunately the discontinuous solution at which he arrives is in fact true only in the case in which the partition coefficient is a constant, and hence the greater part of his paper is invalidated. His equation for the rate of movement of the band reduces to the same as our equation (2) when the partition coefficient is constant; but as it stands it implies the absurd result that a square-fronted band is propagated unchanged while a band of any other shape changes shape during development.

It is undoubtedly possible to make closer allowance for the effects of diffusion than we have done here, but the simple picture of the working of the chromato-

gram is apt to be lost, and no very close approximation to practice can be obtained owing to the lack of constancy of the partition coefficient.

By employing the equivalent theoretical plate concept we are enabled to compare the chromatogram with other 'column' devices, and the astonishingly high efficiency of the chromatogram is made apparent. The H.E.T.P. is of the order of 1 cm. for the best distillation columns [cf. Podbielniak, 1941] and about the same for extraction columns [cf. Ney & Lochte, 1941]. As shown below, the H.E.T.P. in our chromatograms is of the order of 0·002 cm.

2. APPLICATION TO THE MICRO-DETERMINATION OF THE HIGHER MONOAMINO-ACIDS IN PROTEINS

Description of the chromatogram

Silica ('pure precipitated' B.D.H.) was boiled repeatedly with conc. HCl (which removed much iron etc.), then washed with distilled water and finally extracted with warm 97% alcohol, which completed the removal of iron and removed some fatty material. The gel was then dried at 110° and was stored in a closed vessel.[1]

The gel was prepared for the chromatogram by adding 70% *w/w* of a saturated solution of methyl orange in water. The solution is added to the gel at once, and thoroughly mixed in. A dry pink powder results, which barely adheres to the walls of the vessel in which it is mixed. An amount of this material containing 5 g. of silica is then suspended with stirring in about 35 ml. of chloroform saturated with water and containing 1% *v/v* of *n*-butyl alcohol. At this point the colour of the gel changes from pink to yellow. The suspension is poured into a chromatogram tube (int. diam. 1 cm.; length 30 cm., furnished at its bottom with a double layer of filter paper mounted on a perforated silver plate). The gel packs down as the chloroform flows out at the bottom of the tube; being lighter than the chloroform it floats in it. When it has packed down to its final position, the top surface appears to be dry, and if evaporation is prevented, no further chloroform leaves the column, capillary forces preventing the entry of air. The gel does not float up again when fresh chloroform is carefully added at the top of the column. The solvent emerging at the bottom of the column is almost free from indicator, the methyl orange being firmly held in the aqueous phase.

The substances for analysis are dissolved in a little chloroform-butyl alcohol, and this solution is carefully added to the top of the column by allowing it to run from a pipette down the side of the chromatogram tube. When this addition and any washings have drained into the gel, the chromatogram is developed by adding fresh solvent at the top of the column.

The position of the acids is revealed by the indicator, which turns from yellow through orange to pink. As development proceeds the single pink band

[1] Owing to the variability of the B.D.H. silica, difficulty has been found in repeating the preparation given above, but the following method gives a reproducible and satisfactory material. Commercial waterglass (140° Tw—Jos. Crossfield, Ltd., Warrington) is diluted with 2 vols. water, and 10 *N* HCl is stirred in, with methyl orange as internal indicator. After standing several hours (more acid being added if required) it is filtered and washed with distilled water on a Buchner till washings are free from indicator. The gel is allowed to age, wet, for 1–2 days on the filter, and after further washing dried at 110°. For use in the chromatogram 50% *w/w* of indicator solution is added to the gel.

at the top of the column resolves into constituent bands which move down the column at characteristic rates.

The movement of the acetamino-acids studied is powerfully affected by minor constituents of the solvent mixture. If the chromatogram is run with pure chloroform, the acids move extremely slowly and show little separation. This may be attributed to adsorption on the silica. When B.P. chloroform (containing 1 % of ethyl alcohol) is used, the acids move readily, presumably because they are eluted from the silica by the alcohol. The separation and recovery of acetamino-acids depend on the nature and quantity of the alcohol added to the chloroform. We have had our best separations with chloroform containing 0·5 % of *n*-butyl alcohol, but the improved recovery obtained when the butyl alcohol content is raised to 1 % has caused us to use this mixture for routine work. With B.P. chloroform, recoveries of about 70 % were obtained, with good separation.

It is possible, using 0·5 % butyl alcohol, to separate as their acetyl derivatives phenylalanine, *nor*leucine, *iso*leucine, leucine, proline and valine. With 1 % butyl alcohol the naturally occurring amino-acids separate into three groups, within which separation is imperfect: (i) phenylalanine; (ii) leucine-*iso*leucine; (iii) proline-valine-methionine.

The acetyl derivatives of alanine, glycine etc. remain at the top of the column, as slowly moving bands separated by a wide space from the substances mentioned above.

Test of relationship between rate of movement of band and partition coefficient

A column was started using a solution of 2 mg. each of acetyl-*l*-proline hydrate and acetyl-*dl*-phenylalanine. The column had 1 cm. diameter and length 20 cm. The column was developed with chloroform + 1 % *v/v* of *n*-butyl alcohol until the bands were well separated. The movement of the centre of each band for a given movement of the liquid surface above the column was then measured.

The column contained 5 g. of dry silica gel, 3·5 ml. of water and 10 ml. of chloroform phase. Hence, assuming the density of silica to be 2·3 g./ml., $A_I = 0{\cdot}11$ cm.^{2}, $A_S = 0{\cdot}175$ cm.^{2}, and $A_L = 0{\cdot}5$ cm.^{2}

From equation (2) the value of the partition coefficient may be calculated:

$$\alpha = \frac{A}{RA_S} - \frac{A_L}{A_S} = \frac{4{\cdot}5}{R} - 2{\cdot}8.$$

In Table 1 the values of α found thus, and by direct titration of the two phases after shaking together in a separating funnel are given for the two acetamino-acids:

Table 1

Acetamino-acid	R	α (determined from band rate)	α (determined directly)	c (mg. solute per ml. aqueous phase in direct determination)
Acetylproline	0·37	9·4	9·5	10
Acetylphenylalanine	1·07	1·4	1·3	2

The values for α as determined by the two methods are in good agreement. A comparison of the values given here with those given by Synge [1939] and by Martin & Synge [1941, 1] shows the great effect on the partition coefficient of the addition of 1 % of butyl alcohol.

In all cases so far investigated the rate of movement of the band becomes less the more dilute the solution, i.e. the partition coefficient becomes smaller

in strong solution [cf. Martin & Synge, 1941, 1]. The concentration must of course be similar to obtain concordant results by the two methods of determining the partition coefficient.

Measurement of H.E.T.P. for column

An estimate of the value of h was obtained from the width of an acetyl-*dl*-valine band in the course of its development. Fig. 3 shows the width of the band as measured, and the width calculated, assuming that $h=0{\cdot}002$ cm. and that the visual edge of the band occurred at $r \pm 2(\sqrt{r+w})$, where $2w$ is the initial width of the band as measured before development. The curves have been fitted to the appropriate time base to show the constancy of the rate of travel of the band.

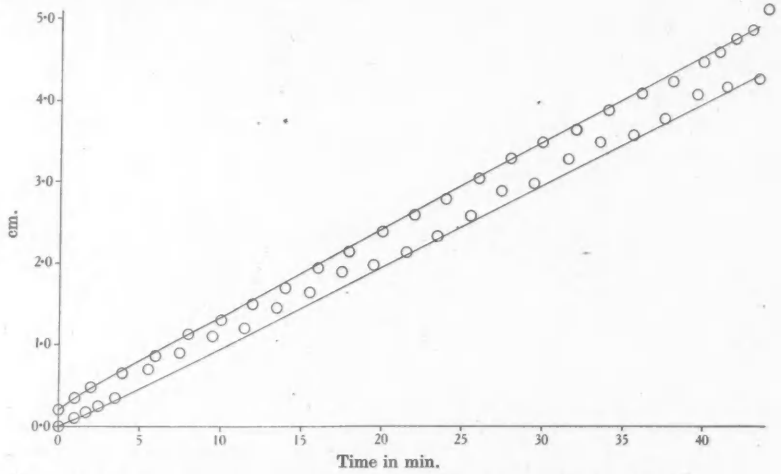

Fig. 3. Development of acetylvaline band. The points show the position of the band edges during development. The lines show calculated width.

There are three sources of error to be remembered here. The edge of the band is really measured at a definite concentration. This tends to make the width as measured less than the true value at high dilutions. Variation of the partition coefficient with concentration and diffusion from plate to plate increase the width of the band over that predicted by theory.

Though the value of h given above cannot be claimed as accurate, it is clearly of the right order.

Use of chromatogram for quantitative analysis

Tests with individual acetamino-acids showed that good recoveries were obtained from the chromatogram when used as described above, and it has therefore been adapted to the analysis of protein hydrolysates for these amino-acids. The preparation of the extract for the chromatogram is based on our earlier work [Martin & Synge, 1941, 1].

About 100 mg. of protein are hydrolysed as usual by refluxing for 24 hr. in 6N HCl, and the bulk of the HCl is removed by repeated evaporation with water *in vacuo*. The material thus obtained is taken up in water and made

alkaline to thymolphthalein by addition of 6*N* NaOH and is then concentrated *in vacuo* to a thin syrup. This is acetylated by addition of 10 ml. of 2*N* NaOH and 1 ml. of acetic anhydride in five equal portions in the course of 15 min. Between each addition the reaction vessel is thoroughly shaken and cooled with ice-water. After this the mixture is allowed to remain alkaline to thymolphthalein for 10 min.; 1 ml. of 10*N* H_2SO_4 is then added, and the solution is concentrated *in vacuo* below 40° to about 5 ml. It is then acidified to thymol blue by careful addition of further 10*N* H_2SO_4 (strong red colour with indicator). The solution is transferred to a separating funnel at a volume of 10 ml. with water, and is extracted with five successive batches of 50 ml. of chloroform. The chloroform extracts are filtered through paper into a distilling flask and are concentrated first at atmospheric pressure and finally *in vacuo* leaving a residue of acetamino-acids which is made up in alcohol to 10 ml. 3 ml. of this alcoholic solution (corresponding to about 30 mg. of protein) are evaporated to dryness *in vacuo* in a small Erlenmeyer flask, and stored overnight over H_2SO_4 and soda-lime in a vacuum desiccator, to effect removal of free acetic acid. The material in the flask is then transferred quantitatively to the chromatogram column in a minimum of chloroform-butyl alcohol, and the chromatogram is developed as described above with chloroform saturated with water and containing 1 % of butyl alcohol.

As each of the three main bands passes out of the bottom of the tube, the chloroform receiver is changed. The solution collected is evaporated *in vacuo*, and the residue is taken up in a little water and titrated with 0·01*N* $Ba(OH)_2$, using phenolphthalein as indicator. Care is taken to exclude CO_2 during the titration.

As a test of the method we employed the artificial mixture of ammonia and 14 amino-acids previously used by us [Martin & Synge, 1941, 2: Table 2]. This mixture had been refluxed with HCl, and was worked up exactly as if it were a protein hydrolysate. In a second experiment *l*-proline was added to the mixture. The results of these experiments are shown in Table 2.

Table 2. *Determination of amino-acids in artificial mixtures*

(Figures are N in % of total N of original mixture)

	Exp. I			Exp. II		
Amino-acid	Found	Calc.	Recovery %	Found	Calc.	Recovery %
Phenylalanine	2·1	2·6	81	1·9	2·4	79
Leucine	9·6	10·3	93	9·5	9·7	98
Proline	6·6	0·0	110	11·4	5·7	101
Valine		4·7			4·4	
Methionine		1·3			1·2	

In Exp. I material corresponding to 7·6 mg. N of the original mixture was put on the column. This quantity was excessive and caused broadening of the bands, with imperfect separation, which is reflected in the raising of the valine-methionine figure at the expense of the leucine figure. In Exp. II, material corresponding to 5·0 mg. N was put on, giving visually satisfactory separation of the bands, and this is reflected in the improved accuracy of the results.

We have also tested the method on a hydrolysate of the same sample of Merino 64's wool which we have previously analysed [Martin & Synge, 1941, 1] for the amino-acids in question. An amount of extract derived from wool containing 4·8 mg. of N was used for the determination. The results are compared with our previous figures in Table 3.

The reason for the lower recovery of phenylalanine than of the other amino-acids has not yet been explained. The control experiments described above were carried out under the conditions giving the best yields which we have so far obtained, i.e. with 1% of butyl alcohol in the chloroform. When the chromatogram is being used for the qualitative investigation of preparations, e.g. for homogeneity, and in preparative work, the use of chloroform containing a lower percentage of butyl alcohol gives superior separations.

Table 3. *Determination of higher monoamino-acids in Merino 64's wool*

(Figures are N in % of total N of wool)

Amino-acid	Present determination	Previous determination
Phenylalanine	1·9	1·9
Leucine-*iso*leucine	7·4	7·2
Proline	8·2	4·9
Valine		3·4
Methionine		0·4

The above determinations have been carried out on quantities of material corresponding to about 30 mg. of protein. By using a narrower chromatogram tube and micro-titration technique it is obvious that smaller quantities could be analysed with equal accuracy.

Summary

1. A new form of chromatogram is described, depending not on adsorption on a solid phase, but on partition of solutes between two liquid phases.

2. Visual detection of colourless acids in this chromatogram can be achieved by adding an indicator to one of the phases.

3. A general theory of chromatography applicable to substances with linear distribution isotherms is developed. This theory is based on the 'theoretical plate' concept, thus directly relating chromatography to fractional distillation and extraction.

4. The theory is checked experimentally in the new chromatogram.

5. The new chromatogram is applied to the micro-determination of the higher monoamino-acids in protein hydrolysates.

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152. A STUDY OF THE PARTIAL ACID HYDROLYSIS OF SOME PROTEINS, WITH SPECIAL REFERENCE TO THE MODE OF LINKAGE OF THE BASIC AMINO-ACIDS

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The classical Hofmeister-Fischer hypothesis of the peptide structure of proteins to-day finds almost universal acceptance as a basis of chemical study. This implies that proteins for the most part are built up of peptide chains of the type

—CONH.CH*R*.CONH.CH*R'*.CONH.CH*R''*—,

where *R*, *R'*, *R''* are the side-chains of the different amino-acids, of which only 20 odd molecular species have been isolated so far from the whole range of living organisms. It seems clear, therefore, that the distinctive properties of proteins reside in the arrangement rather than the nature of the amino-acid residues.

Bergmann & Niemann [1936; 1937; 1938] have put forward a theory of protein structure based on the idea that amino-acid residues are repeated at regular intervals along the peptide chain, and, further, that the 'frequency' of such recurrency must be factorizable entirely by 2 and/or 3, as must the total number of amino-acid residues in the molecule, and therefore the total number of residues of each amino-acid species in the molecule.

This hypothesis imposes considerable limitations on the astronomical possibilities of protein isomerism permitted by the Hofmeister-Fischer hypothesis. The main positive chemical evidence presented in its favour by Bergmann and others has been the results of amino-acid analysis of complete hydrolysates of proteins. It is clear that the only direct evidence in favour of a hypothesis concerning the order of amino-acid residues in a peptide chain can come from the isolation and identification of fragments of this chain containing at least two amino-acid residues.

Since n molecular species of simple amino-acids can only give rise to n^2 molecular species of dipeptides, an investigation of the dipeptides resulting from the partial hydrolysis of proteins should be possible technically, and, if we can be sure that the dipeptides studied do not arise by rearrangement, but are true fragments of the original peptide chain of the protein, the characterization of these compounds should yield information as to the structure of the original protein molecule.

A considerable body of data of this sort has been accumulated during the past 40 years. In the course of an examination of this literature undertaken by one of us (R. L. M. S.), special attention was paid to possible rearrangements occurring in the course of preparing and isolating the products of partial hydrolysis. No evidence could be found in the literature suggesting that partial

hydrolysis of proteins by strong mineral acid in the cold was likely to give rise to rearrangement or ring-formation in peptides such as might be expected to result from enzymic hydrolysis [Behrens & Bergmann, 1939], or from the action of hot dilute acids [Abderhalden & Funk, 1907; Abderhalden & Komm, 1924, 1, 2].

Cold concentrated acid has accordingly been used as the hydrolytic agent in the work described below. The possibility that the peptides studied result by rearrangement of previously existing structures has not been rigorously excluded: to devise a satisfactory control experiment for this purpose is not simple. The best control would seem to be to find a physically well-characterized and homogeneous protein containing only one residue of a particular amino-acid species per molecule. If this protein, as the physical and chemical evidence so far available strongly suggests, is a definite chemical compound in the same sense as are organic compounds of low molecular weight, then only two di-peptides of the single amino-acid residue in question should be isolable from a partial hydrolysate of the protein.

This would be the case if the protein had a definite chemical structure, irrespective of whether it had any particular structure, e.g. one conforming to the Bergmann-Niemann hypothesis. We have begun to undertake work in this direction, but as it is likely to be extremely protracted, it seems desirable to publish the evidence so far obtained on protein partial hydrolysis products, particularly since we have been unable to find any reference in the literature suggesting the rearrangement of peptide bonds under the hydrolytic conditions which we have employed.

The work here described falls into two sections:

I. A study of the course of hydrolysis of some proteins by cold concentrated hydrochloric acid, with special reference to the lability of the different types of bond present.

II. A study of the nature of the peptides containing basic amino-acids, which result from such hydrolysis.

Materials used in the investigation

In addition to wool, we have studied a preparation of edestin given to us by Prof. A. C. Chibnall, since for this protein his stoichiometric evidence seems better than any presented for other proteins in favour of the Bergmann-Niemann hypothesis. We have also studied gelatin (Coignet Gold Label) since current theories [cf. Bergmann, 1935; Bergmann & Stein, 1939; Astbury, 1940] assign to this substance a rather definite structure.

Analytical methods used

Estimation of cystine. Use has been made of the method of Sullivan [cf. Block, 1938, 2]. We have worked on a quarter the scale described in Block's book, and have employed direct visual comparison of the test with the standard solutions.

As cysteine residues involved in peptide linkage are not thought to give a colour in this reaction, it was important to show that the alkaline conditions employed did not give rise to significant hydrolysis of peptide bonds. For this purpose a mixed peptide solution of known amino-N content was kept with alkali of the same strength for the time usually allowed for the Sullivan reaction. A redetermination of amino-N showed no significant increase.

Estimation of arginine [cf. Kossel & Gross, 1924]. This has been done by the precipitation of arginine as its monoflavianate by addition of approx. 1·7 mol. of flavianic acid per mol. of arginine to the weakly acid unknown solution. The method was only applied to solutions known to be rich in arginine. The results are likely to be high owing to contamination with histidine, which, however, is a minor constituent of the proteins studied. The precipitation mixture was kept overnight at 0° and was then filtered through a weighed sintered glass funnel. The precipitate was washed with a little aqueous 0·5% flavianic acid and finally with alcohol, and then dried to constant weight *in vacuo* at room temperature over H_2SO_4. Control experiments with mixtures of arginine and alanine are described in § II below.

Estimation of higher monoamino-acids and proline. By acetylating a protein hydrolysate and extracting the acetylated mixture with chloroform under standard conditions it has proved possible [cf. Synge, 1939, 1; Martin & Synge, 1941, 1; Martin & Synge, accompanying paper] to obtain on the microscale a measure of the amount of these amino-acids present.

The work with the higher monoamino-acids described in the present paper was carried out before the liquid-liquid chromatogram [cf. Martin & Synge, accompanying paper] had been adapted for quantitative use. Accordingly use was made of total N (Kjeldahl) determinations on the chloroform extract prepared as described in the accompanying paper for obtaining an estimate of the aggregate of higher monoamino-acids present. The extract was also hydrolysed (by refluxing for 3 hr. in 2*N* HCl after it had been freed from alcohol by evaporation) and subjected to a Van Slyke amino-N determination (4 min. reaction time). The resulting figure gave an estimate of the higher monoamino-acids other than proline [cf. Van Slyke, 1911; Synge, 1939, 1]. The extract was subjected to qualitative investigation in the chromatogram, using B.P. chloroform saturated with water as the developing solvent.

Estimation of total hydroxyamino-acids and threonine (also free ammonia). These have been determined as ammonia and acetaldehyde, respectively, liberated by periodic acid, as described by Martin & Synge [1941, 2]. After the commencement of the present work the superior techniques of Nicolet & Shinn [1941] for serine and of Shinn & Nicolet [1941] for threonine were published. For the sake of uniformity we have not modified our procedure in the course of the work, the yields of ammonia ('total hydroxyamino-acids') and acetaldehyde ('threonine') being recorded 'corrected' throughout this paper, on the assumption that they are 75 and 70% respectively [cf. Martin & Synge, 1941, 2]. Free ammonia was determined in the course of the total hydroxyamino-acid determination.

Estimation of amino-N. The manometric Van Slyke procedure (4 min. reaction time) was employed. Where the sample contained free mineral acid this was neutralized before analysis with excess of sodium acetate.

Estimation of total N. Semi-micro-Kjeldahl determinations were employed, using the incineration mixture of Campbell & Hanna [1937].

Estimation of free amino-acids. The ninhydrin procedure of Christensen *et al.* [1941] was employed.

Estimation of tyrosine. The method of Arnow [cf. Block, 1938, 2] was employed.

I. The course of hydrolysis of some proteins by cold concentrated hydrochloric acid, with special reference to the lability of the different types of bond present

Partial hydrolysis has been effected in all the work described in this paper by solution of the proteins in an excess of 10*N* HCl at 37°. Wool dissolves almost entirely in the course of the first 24 hr. if gently shaken at intervals, leaving a particulate residue which is mostly ether-soluble. Edestin and gelatin give an immediate clear solution; in the case of gelatin, a small amount of amorphous material is deposited during the first 2 days' hydrolysis. Wool and edestin hydrolysates show a brilliant purple colour which after 3–4 days darkens to a clear brown. Hydrolysis was effectively stopped at any desired stage by repeated evaporation of the mixture with water *in vacuo* below 40°, followed by dilution to large volume and storage at 0°.

The above-mentioned determinations were made on both the partial and the complete hydrolysates. The latter were prepared by refluxing the material in 6*N* HCl for 24 hr.

The results obtained are tabulated below (Tables 1–6). Fig. 1 shows the kinetics of each determination for the partial hydrolysate of Merino 64's, the results of each determination on the partial hydrolysate being expressed as 'B values' (see tables). Fig. 2 (same units) compares the kinetics of evolution of amino-N in the same proteins.

Table 1. *Evolution of amino-groups during partial hydrolysis*

Time of hydrolysis hr.	Wool (Merino 64's)		Wool (flannel)		Edestin		Gelatin	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
19	—	—	—	—	—	—	22·9	39·5
24	26·2	34·1	—	—	—	—	—	—
43	—	—	—	—	—	—	32·1	55·1
48	34·3	44·6	30·4	39·5	23·0	35·9	—	—
96	38·8	50·5	—	—	—	—	—	—
114	—	—	40·0	52·0	—	—	—	—
120	—	—	—	—	32·4	50·6	—	—
139	—	—	—	—	—	—	44·9	77·0
166	—	—	46·5	60·3	37·0	57·8	—	—
192	48·3	62·9	—	—	—	—	—	—
Complete	76·8	(100)	77·0	(100)	64·0	(100)	58·3	(100)

A values: Amino-N, as percentage of total N of protein.
B values: '*A* value' as percentage of its value in complete hydrolysate.

Table 2. *Evolution of free amino-acids during partial hydrolysis*

Time of hydrolysis hr.	Wool (Merino 64's)		Wool (flannel)		Edestin		Gelatin	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
48	11·9	17·4	—	—	—	—	—	—
96	20·7	30·2	—	—	—	—	—	—
139	—	—	—	—	—	—	33·7	41·4
166	—	—	27·2	39·7	23·4	36·9	—	—
192	25·7	37·4	—	—	—	—	—	—
Complete	68·6	(100)	68·6	(100)	63·4	(100)	81·5	(100)

A values: CO_2 liberated by ninhydrin as N in percentage of total N of protein.
B values: '*A* value' as percentage of its value in complete hydrolysate.

Table 3. *Evolution of amino-groups of hydroxyamino-acids during hydrolysis*

Time of hydrolysis hr.	Wool (Merino 64's)		Wool (flannel)		Edestin		Gelatin	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
24	6·4	42·6	—	—	—	—	—	—
48	9·3	62·0	—	—	—	—	—	—
96	11·0	73·0	—	—	—	—	—	—
139	—	—	—	—	—	—	4·9	94·0
166	—	—	10·2	76·5	7·3	102·0	—	—
192	12·1	80·6	—	—	—	—	—	—
Complete	15·0	(100)	13·2	(100)	7·1	(100)	5·2	(100)

A values: Ammonia-N liberated by periodic acid (corrected) as percentage of total N of protein.
B values: '*A* value' as percentage of its value in complete hydrolysate.

Table 4. *Evolution of amino-groups of threonine during partial hydrolysis*

Time of hydrolysis hr.	Wool (Merino 64's)	
	A	*B*
24	1·9	50
48	2·9	76
96	4·0	105
192	4·3	113
Complete	3·8	(100)

A values: CH_3CHO as N (corrected) in percentage of total N of protein.
B values: '*A* value' as percentage of its value in complete hydrolysate.

Table 5. *Evolution of ammonia during partial hydrolysis*

Time of hydrolysis hr.	Wool (Merino 64's)		Wool (flannel)		Edestin		Gelatin	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
24	5·6	66·0	—	—	—	—	—	—
48	6·5	75·5	—	—	—	—	—	—
96	7·0	81·5	—	—	—	—	—	—
139	—	—	—	—	—	—	0·5	55·0
166	—	—	7·1	86·0	9·4	92·0	—	—
192	7·2	84·0	—	—	—	—	—	—
Complete	8·6	(100)	8·3	(100)	10·2	(100)	0·9	(100)

A values: Ammonia-N as percentage of total N of protein.
B values: '*A* value' as percentage of its value in complete hydrolysate.

Table 6. *Evolution of free cysteine (or '½ cystine') during partial hydrolysis*

Time of hydrolysis hr.	Wool (Merino 64's)		Wool (flannel)		Edestin	
	A	*B*	*A*	*B*	*A*	*B*
24	0·7	8·4	—	—	—	—
48	1·2	15·8	—	—	—	—
96	2·4	29·9	—	—	—	—
166	—	—	4·6	—	0·42	117·0
192	2·9	35·8	—	—	—	—
Complete	7·9	(100)	—	—	0·36	(100)

A values: Cystine-N (Sullivan) as percentage of total N of protein.
B values: '*A* value' as percentage of its value in complete hydrolysate.

Size of peptides formed during partial hydrolysis

From Fig. 1 it is seen that free amino-acids are set free from the outset in the partial hydrolysis of wool, and that after about a week, 37 % of the amino-acid residues are free, the rest being presumably in the form of peptides.

An estimate of the size of the peptides present may be made by comparing the number of peptide bonds split (change of amino-N value) with the number of amino-acid residues liberated (change of ninhydrin-CO_2 value) on complete hydrolysis.

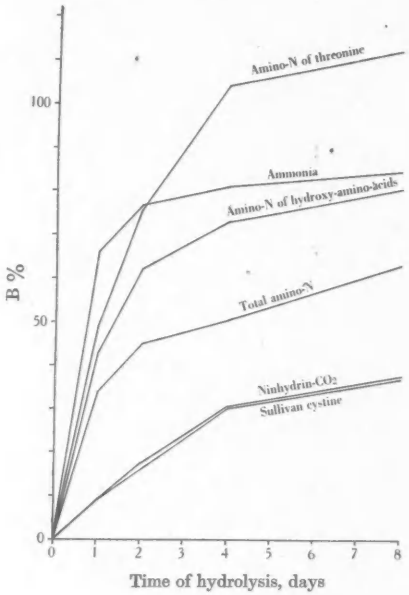


Fig. 1.

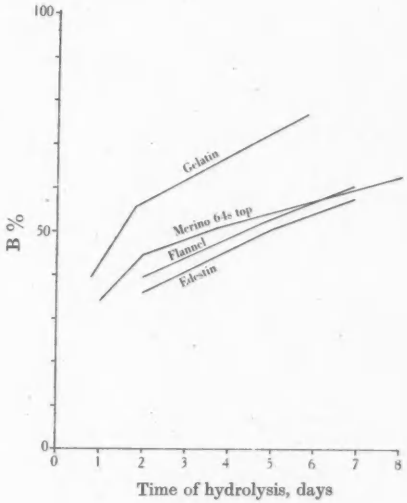


Fig. 2.

Fig. 1. Kinetics of liberation of various groups in partial acid hydrolysis of wool (Merino 64's top). The values are expressed as percentages of the value determined after complete hydrolysis.

Fig. 2. Kinetics of liberation of amino-N in partial acid hydrolysis of various proteins. The values are expressed as percentages of the value determined after complete hydrolysis.

Given for any partial hydrolysate,

$$r = \frac{\Delta\ (\text{'}A\text{ value' for ninhydrin-}CO_2)}{\Delta\ (\text{'}A\text{ value' for amino-N})} \text{ on completing the hydrolysis,}$$

we have for a dipeptide, which for the splitting of 1 bond liberates 2 amino-acid residues, $r = \frac{2}{1} = 2{\cdot}0$, and for a tripeptide $r = \frac{3}{2} = 1{\cdot}5$, and for a tetrapeptide $r = \frac{4}{3} = 1{\cdot}33$.

Table 7 shows the derivation of r values for partial hydrolysates of the 3 proteins investigated.

Table 7

Protein	Time of hydrolysis hr.	Δ_A ninhydrin-CO_2	Δ_A amino-N	r
Wool (Merino 64's)	192	42·9	28·5	1·50
Edestin	166	40·0	27·0	1·48
Gelatin	139	47·8	13·4	3·6

These figures suggest that the peptides present in the partial hydrolysates of wool and edestin are on the average tripeptides.

Since proline and hydroxyproline yield CO_2 with ninhydrin, but do not possess amino-N, the presence of peptide bonds involving the imino-groups of these residues will affect the *r* value obtained, as will deviations of amino-N and ninhydrin-CO_2 values from the 'theoretical' in the case of particular amino-acids and peptides. *r* values should therefore be interpreted with reserve.

The high *r* value for gelatin may be explained by the high proline and hydroxyproline contents. In this substance (cf. Fig. 2) it seems that the bonds involving amino-groups are more readily split by cold concentrated acid than those of wool and edestin, or than the bonds in gelatin itself which involve imino groups.

'Amide-nitrogen'

In the partial hydrolysates the liberation of ammonia is found to proceed rapidly, and to reach a final value lower than that found for 'complete' hydrolysis. The lability of the ammonia agrees with the data of Vickery [1922; cf. Shore *et al.* 1936; Bailey, 1937].

It is suggested that the extra ammonia liberated on further refluxing for 24 hr. in 6*N* HCl is not true 'amide-nitrogen', but arises from the decomposition of hydroxyamino-acids such as serine. From Table 8 it will be seen that this 'extra' ammonia amounts consistently to about 10% of the hydroxyamino-acids present.

Table 8

Protein	Wool (Merino 64's)	Wool (flannel)	Edestin	Gelatin
'Extra' NH_2-N (see Table 5) as percentage of total N of protein	1·4	1·2	0·8	0·4
Hydroxyamino-acid-N (see Table 3) as percentage of total N of protein	15·0	13·2	7·1	5·2
NH_2 as percentage of hydroxyamino-acids	10·0	9·0	11·0	8·0

The destruction of serine residues by hot mineral acid has been noted by Posternak [1927] and by Damodaran & Ramachandran [1941].

Free cysteine or '½-cystine'

In wool, this appears to be liberated during partial hydrolysis with acid at a rate not significantly different from the average rate of liberation of other kinds of amino-acids, as shown by the coincidence of the 'Sullivan' and 'ninhydrin-CO_2' graphs in Fig. 1. In edestin, under the conditions employed, all the cystine appears to be liberated as the free amino-acid.

Liberation of amino-groups of hydroxyamino-acids

The 'total hydroxyamino-acid' and 'threonine' determinations on the partial hydrolysates may be taken as a measure of hydroxyamino-acid residues having their α—NH_2 but not necessarily their —COOH groups free, since periodic acid

appears to react with compounds having $—NH_2$ and —OH groups bound to adjacent C atoms, but not to react when the $—NH_2$ group is acylated [cf. Nicolet & Shinn, 1939]. In the case of hydroxylysine the $\epsilon—NH_2$ group may be expected to react under the same conditions. These determinations may therefore be taken as a measure of the rupture of peptide bonds involving these $—NH_2$ groups. In the case of wool, all those bonds involving threonine residues (Table 4) appear to be readily broken, but not all those involving serine (Table 3). In gelatin and edestin all such bonds appear to be broken under the same conditions. This is also the case for all the derived fractions described in § II of this paper, consisting of basic peptides derived from wool, edestin and gelatin. It seems that, in these proteins at least, the bonds involving the amino-groups of serine and threonine are less than usually resistant to acid hydrolysis. In the field of simple compounds this is paralleled by Abderhalden & Bahn's [1932; 1935] study of the acid hydrolysis of acylated serine derivatives and of diketopiperazines containing serine.

The kinetics of hydrolysis of a peptide chain

It is useful in interpreting the above data to have some physical picture, however imperfect, of the mechanism of the hydrolysis of a protein, a chemical reaction in which one molecular species (the protein) is transformed into (say) 20 (the amino-acids) and in the course of which a number of intermediate substances transitorily appear and disappear. Both the relative amounts and the actual nature of these intermediates one may expect to be very considerably affected by quite small changes in the conditions of hydrolysis. The stability to hydrolysis of any particular peptide bond in a protein molecule will be determined by the nature of the amino-acids involved in it and by the nature of other neighbouring amino-acids, and these considerations may be expected to determine the point at which a long peptide chain containing a variety of amino-acid residues is first split. Once this split has occurred, a free positively charged $—NH_3^+$ or $=NH_2^+$ group is liberated, and this may be expected to repel H^+ ions and thus stabilize to acid hydrolysis the remaining peptide bond in which the amino-acid residue carrying the positively charged group is involved [cf. Moggridge & Neuberger, 1938; Synge, 1939, 2]. Higher peptides will thus have a tendency to be shortened to dipeptides. An analogous mechanism should apply to the negatively charged $—COO^-$ groups set free in alkaline hydrolysis, and it is reasonable to expect dipeptides to exhibit a greater stability than higher peptides both to acid and to alkaline hydrolytic agents. Those peptide bonds, then, in a protein that prove especially resistant to hydrolysis and are therefore recognizable as originally present in the protein through their isolation in simple peptides, may not initially have had any great intrinsic stability but may simply have been preserved through their proximity to a readily hydrolysable bond.

II. The peptides containing basic amino-acids resulting from hydrolysis by cold concentrated hydrochloric acid

In attempting to study the mixture of lower peptides resulting from the partial acid hydrolysis of proteins described in the previous section, it seemed to us desirable to begin with a broad separation based on the net electric charge on the molecule at *p*H 6, since such separation can be accomplished electrophoretically with some theoretical certainty as to the types of molecule that will be transported.

The naturally occurring amino-acid molecules can be classified as:

B (Basic). Arginine, histidine, lysine and hydroxylysine which carry a net positive charge at *p*H 6.

A (Acidic). Glutamic and aspartic acids, which carry a net negative charge at *p*H 6. (After partial hydrolysis as described above, all glutamine and asparagine residues will have been converted to glutamic and aspartic acid residues.)

N (Neutral). The other amino- and imino-acids, which carry no net charge at *p*H 6.

Considering now the six possible types of dipeptide resulting from the combination of these categories of amino-acids, on electrophoresis at *p*H 6:

BB and *BN* will migrate towards the cathode,
BA and *NN* will not migrate,
AA and *AN* will migrate towards the anode.

For a start, we have studied those components of a protein partial hydrolysate which migrate towards the cathode, since apparatus is already available [Albanese, 1940; Foster & Schmidt, 1923] for preparing such a fraction, and at least the free basic amino-acids are known not to be affected by secondary chemical action at the cathode.

Such a fraction is likely to prove of theoretical interest in revealing the mode of linkage of the basic amino-acids in the protein. Special importance has been attached to these in the 'kyrine' hypothesis of Siegfried [1903] and in the more recent 'anlage' concept of Block [1938, 1]. The results of work by Siegfried, Levene, Grassmann and others on the 'kyrine' question are of significance in connexion with the present work, as are also other investigations of basic peptides of protein origin, among which those of Kossel, Dirr, Felix and collaborators on partial hydrolysis products of clupein are outstanding.

All this earlier work on basic peptides made use of empirical precipitation methods; in the present work it will be seen that by use of a method based on theory we have succeeded in isolating from partial acid hydrolysates of proteins fractions consisting almost entirely of basic peptides and amino-acids, and containing a high proportion of the total basic amino-acid residues of the original proteins.

Experimental

The electrodialysis cell used in this work was similar to that described by Albanese [1940]. Some simplification was gained by making the cooling tubes reciprocate so as to stir all three compartments. It was found possible to use parchment paper for both the membranes as there was no very serious electrosmotic transfer of the compartment content. The drainage holes used by Albanese were omitted, the liquids being transferred by pipette. The various sections of the cell including the rubber gaskets were coated with paraffin wax to ensure that they were inert towards aqueous solutions. Either an ammeter or a milliammeter could be included in the circuit by means of a two-way switch. The source of current was a CuO rectifier giving 175 V. The circuit was stabilized by insertion of a 200 W. lamp. The cathode of the cell was made of platinum foil, and the anode was of gas carbon.

The behaviour of the cell with mixtures of arginine and alanine was studied, since arginine could readily be determined by flavianic acid as described above,

while alanine could be estimated by a Van Slyke amino-N determination on the filtrate and non-alcoholic washings from the arginine flavianate. A control experiment with arginine alone showed that these carried away a negligible amount of arginine amino-N.

In the first experiment, the middle compartment of the cell contained initially 100 ml. of an aqueous solution containing 1 g. of *dl*-alanine and 1 g. of *l*-arginine hydrochloride. The anode and cathode compartments each contained 100 ml. of distilled water. The current rose and fell again in the course of 3 hr. No attempt was made to control the *p*H of the middle compartment. At the end of the run, no arginine and no Cl^- could be detected in the middle compartment; 101 % of arginine flavianate was recovered from the catholyte, and 39 % of the alanine used was found also to be present.

A second experiment was conducted using 0·5 g. of *dl*-alanine and 0·5 g. of *l*-arginine hydrochloride in the middle compartment, the *p*H of this mixture being first adjusted to 7 with NaOH. During the run the *p*H of the middle compartment was not controlled and at the end of the run the middle compartment was again found to be free from arginine and Cl^-. This time 19 % of the alanine was transferred to the catholyte.

In a third experiment the same initial solution in the middle compartment was maintained throughout the run at *p*H 6 by addition of NH_3 (bromothymol blue used as external indicator). After 80 min. the current fell, and all the arginine and only 7·3 % of the alanine were found in the catholyte after removal of NH_3 by evaporation *in vacuo*.

Next the rate of diffusion between the compartments of the cell was studied. 100 ml. of water containing 0·5 g. of alanine were placed in the middle compartment, and 100 ml. of water in each of the end compartments. After 1·5 hr. stirring without passage of current 5 % of the alanine had entered the cathode compartment, and after 3 hr. 9·7 % of the alanine had entered.

The transfer of alanine in the third experiment to the cathode compartment is thus explicable in terms of diffusion, to which must be added transport in the electrosmotic stream (about 3 ml. per hr.) from the middle to the cathode compartment. It is probably this unidirectional flow of liquid through the membrane which prevents the electrodialytic return of alanine from the catholyte to the middle compartment, for in the weakly alkaline catholyte alanine residues carry a net negative charge.

The electrodialytic fractionation of partial protein hydrolysates

By thrice-repeated electrodialysis, it has proved possible, in the light of the control experiments just described, to separate the basic peptides and amino-acids of partial protein hydrolysates from nearly all neutral and acid constituents.

As an example of the procedure which we have evolved, we describe in detail the fractionation of a partial hydrolysate of gelatin: 50 ml. of the solution to be fractionated, containing 197 mg. of N, were put in the middle compartment of the cell. This solution still contained a little of the HCl used to hydrolyse the gelatin, although most of this was already removed (see § I). All the compartments were made up to 100 ml. with distilled water, cooling and stirring were commenced and the current was switched on. It remained at a few mA. until after the addition of a few drops of NH_3 (sp. gr. 0·880) to the catholyte and of 10*N* HCl to the anolyte, thus raising the conductivity of the end compartments. The current rose sharply to about 80 mA., then steadily until after $2\frac{3}{4}$ hr. it had reached 450 mA. It then fell for $1\frac{1}{4}$ hr. to 200 mA.; previous

experiments having shown that no further fall was to be expected, the contents of the cathode compartment were transferred to a Claisen flask and concentrated *in vacuo* below 40°. When the volume was about 50 ml. the solution (which reacted in the alkaline range of thymol blue) was neutralized to bromothymol blue with 2*N* H_2SO_4. Great care was taken to add no more H_2SO_4 than was necessary just to shift the indicator from its alkaline colour. The solution was then put back in the middle compartment of the cell, and all compartments brought to 100 ml. with water as before. The run was conducted as before except that every 2–3 min. the *p*H of the middle compartment was inspected. It fell steadily during the run, but was all the time on the range of bromothymol blue. The current rose to 225 mA., and after 31 min. had fallen to 31 mA. The contents of the cathode compartment were evaporated, neutralized and returned to the cell exactly as before, and the next electrodialysis was conducted in the same way, except that this time one drop of conc. NH_3 was required to keep the middle compartment solution on the range of the indicator. The current rose to 270 mA. and then fell to 20 mA. after 31 min. The amino-N content of the middle compartment was then determined, and it was found to be 0·97% of the amino-N originally taken for the fractionation. As this was satisfactorily low, the contents of the cathode compartment were evaporated as usual to remove NH_3, then neutralized to *p*H 5 with HCl and made up to 50 ml. in a volumetric flask.

Electrodialysis of a complete protein hydrolysate

This served as a control of contamination of the 'basic peptide fraction' by neutral substances. Wool (Merino 64's) was hydrolysed completely by refluxing for 24 hr. in 6*N* HCl. The excess HCl was removed as usual by evaporation, and a solution containing 128 mg. N was taken for electrodialytic fractionation exactly as described above; 2% of the amino-N originally taken was found in the middle compartment at the end of the third run. The catholyte contained 25·4% of the N of the wool, of which 34·3% was amino-N. Hydroxyamino-acids, tyrosine and cystine were determined on the catholyte, as shown in Table 9. From this it can be seen that the electrodialysis procedure employed gave a satisfactory elimination of neutral contaminants from the basic fraction.

Table 9

Determination	N as % of N of catholyte	Total amount of given amino-acid in catholyte as % of amount originally present in unfractionated hydrolysate
Total hydroxyamino-acids	0·1	0·6
Tyrosine	0·04	0·5
Cystine (Sullivan)	0·08	0·25

The 'basic peptide fractions' from partial protein hydrolysates

The neutralized 'basic peptide' fraction, made up to 50 ml. as described above, was then investigated as follows.

Some of the solution was kept for determinations to be carried out on the unhydrolysed peptides (amino-N, ninhydrin-CO_2, Sullivan, etc.) and the rest was refluxed for 24 hr. in 6*N* HCl to effect complete hydrolysis, before carrying out further determinations.

Table 10 shows comparative data on the various 'basic peptide fractions' before and after hydrolysis.

Table 10. *Determinations on 'basic peptide fractions' before and after hydrolysis*

Determination	Wool I	Wool II	Edestin I	Edestin II	Gelatin I	Gelatin II
N of basic peptide fraction as percentage of N of original protein	27·1	25·0	33·7	29·1	25·2	22·7
Amino-N as percentage of total N of basic peptide fraction before hydrolysis	23·7	25·6	—	25·1	28·5	28·6
Same after hydrolysis	43·3	45·8	41·0	40·0	47·0	—
Ninhydrin-CO_2 as N in percentage of N of basic peptide fraction before hydrolysis	9·9	8·9	—	9·9	11·0	—
Same after hydrolysis	54·0	47·2	—	39·4	46·3	—
Δ ninhydrin-CO_2	44·1	38·3	—	29·5	35·3	—
Δ amino-N	19·6	20·2	—	14·9	18·5	—
R	2·25	1·90	—	1·98	1·91	—
Cystine-N (Sullivan) as percentage of N of basic peptide fraction before hydrolysis	0·83	—	—	0·06	—	—
Same after hydrolysis	—	2·9	—	0·02	—	—
Total hydroxyamino-acid-N as percentage of N of basic peptide fraction before hydrolysis	—	7·7	—	3·4	3·3	—
Same after hydrolysis	5·5	5·1	2·7	2·0	3·1	—

The fractions described in Tables 10 and 11 were prepared by electrodialysis from partial hydrolysates of proteins in 20 parts of 10*N* HCl at 37° for the undermentioned lengths of time:

Wool I. Commercial Merino 64's top: 192 hr.
Wool II. A commercial loosely woven pure wool flannel: 166 hr.
Edestin I. A preparation of edestin (from Prof. A. C. Chibnall): 168 hr.
Edestin II. The same material hydrolysed on a different occasion for 167 hr.
Gelatin I. Coignet 'Gold Label' gelatin hydrolysed for 139 hr.
Gelatin II. The same hydrolysate subjected to a separate fractionation.

Table 11 shows the individual amino-acids determined in the hydrolysates of the basic peptide fractions. Corresponding determinations on unfractionated complete hydrolysates of the same proteins are shown in Table 12.

Table 11. *Amino-acid determination on hydrolysates of 'basic peptide fractions'*

Determination (N as % N of fraction)	Wool I	Wool II	Edestin I	Edestin II	Gelatin I
Ammonia-N	1·2	2·3	0·7	0·1	0·3
Arginine-N	59·7	—	70·4	—	53·2
Cystine-N (Sullivan)	—	2·9	—	0·0	—
Tyrosine-N	1·7	1·6	1·0	1·1	—
Total hydroxyamino-acid-N	5·5	5·1	2·7	2·0	3·1
'Higher monoamino-acid-N'	10·6	10·6	11·1	9·0	5·8
'Proline-N'	5·1	3·8	0·7	3·1	4·2

Table 12. *Amino-acid determinations on complete protein hydrolysates*

Determination (N as % N of protein)	Wool I	Edestin	Gelatin
Cystine-N (Sullivan)	7·9	0·36	—
Tyrosine-N	2·1	1·3	—
Total hydroxyamino-acid-N	12·2	7·0	5·2
'Higher monoamino-acid-N'	15·3	12·6	9·2
'Proline-N'	7·1	4·1	11·4

The mixture of acetamino-acids obtained in each case after acetylation and chloroform extraction as described above was investigated qualitatively by the chromatographic method already mentioned, using a column about 1 cm. in diameter. An amount of extract containing 1 mg. of N was put on the column, and the mixture of acetamino-acids obtained from the hydrolysate of the complete protein was run at the same time as the mixture obtained from the hydrolysate of the 'basic peptide fraction' of the same protein. In no case could any differences in the sets of bands present be observed. In the case of wool, the relative intensities of the bands were similar in the two columns. With edestin, the leucine-*iso*leucine complex band and the valine band were more intense for the 'basic peptide fraction' than for the whole protein, while the proline band was slightly diminished. With gelatin, the proline and alanine bands of the material from the 'basic peptide fraction' appeared enriched, and the leucine and phenylalanine bands diminished.

Discussion

Yield of basic amino-acids in 'basic peptide fractions'

Table 13 illustrates the proportion of the total arginine residues of the proteins recovered in the 'basic peptide fractions'.

Table 13. *Recovery of arginine in 'basic peptide fractions'*

	Wool	Edestin	Gelatin
Arginine-N as percentage of N of 'basic peptide fraction' (cf. Table 12) $=A$	59·7	70·4	53·2
N of 'basic peptide fraction' as percentage of N of protein (cf. Table 10) $=B$	27·1	33·7	25·2
N of arginine in 'basic peptide fraction' as percentage of protein: $(A \times B)/100 = C$	16·2	23·7	13·4
N of total arginine of protein as percentage of N of protein (cf. Table 15) $=D$	20·2	28·1	14·6
Percentage of arginine residues of protein present in 'basic peptide fraction': $(C \times 100)/D$	80·0	84·0	92·0

It is seen that this recovery ranges from 80 to 92%. The unrecovered arginine sets an upper limit to the proportion of arginine bound in neutral and/or acidic peptides such as arginylglutamic acid [cf. Levene & Birchard, 1912]. Although the recovery of lysine and histidine has not been determined, the total N of the basic peptide fractions is compatible with a similar recovery of these residues, and it is apparent that in the present work we are dealing with the major proportion of the basic amino-acid residues of the proteins studied.

Character of the peptides present in the 'basic peptide fractions'

The use of the ratio r as a measure of the peptide size in a mixture of amino-acids and peptides has been explained in § I of this paper. Table 10 shows the value of this ratio for the 'basic peptide fractions' obtained from the proteins studied, and it suggests that the peptides present are almost entirely dipeptides. As already mentioned, this ratio is also affected by the number of proline and hydroxyproline residues involved in peptide linkage through their imino groups, since they evolve no amino-N on liberation, while becoming susceptible to decarboxylation by ninhydrin. Thus the presence of lysylprolylglycine ($r=3$) in the 'basic peptide fraction' from gelatin (cf. Grassmann & Riederle's [1936] isolation of this from a partial acid hydrolysate of gelatin)

would permit the presence of a proportion of tripeptides containing amino- rather than imino-groups in peptide linkage, while r remains approximately 2. Thus for gelatin, on account of its high content of proline and hydroxyproline, it is less certain than for wool and edestin that a majority of the peptides of the basic fraction are dipeptides.

Relative stability to acid hydrolysis of bonds in proteins involving basic and other amino-acid residues

Siegfried [1903], in putting forward the 'kyrine' hypothesis of protein structure, suggested that the basic amino-acids were bound in a manner specially resistant to acid hydrolysis.

From the present work it is possible to estimate the ratio $\frac{\text{free basic residues}}{\text{total basic residues}}$ in the various partial protein hydrolysates, and to compare it with the corresponding figure for neutral and acidic residues taken together. This is done as follows.

The 'ninhydrin-CO_2' figure for the unhydrolysed 'basic peptide fraction' represents (1 amino-acid residue being equivalent to 1 mol. of ninhydrin-CO_2 expressed as 1 atom N in % of the total N of the protein) the free basic amino-acids in the partial hydrolysate, when allowance has been made (by subtracting the Sullivan value for the unhydrolysed 'basic peptide fraction') for 'free $\frac{1}{2}$-cystine residues' (see following paragraph). This value is shown as A in Table 14. The total basic residues of the protein are known from already existing analyses, and are expressed as N in % of total N in the protein in Table 15. To render them comparable with A, the arginine figures are divided by 4, lysine by 2 and histidine by 3, and the total of basic residues thus arrived at is shown in Table 14 as B.

Table 14. *Relative stability of basic and other residues to acid hydrolysis*

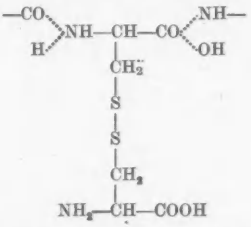
Determination	Wool	Edestin	Gelatin
'Ninhydrin-CO_2' of unhydrolysed 'basic peptide fraction' as N in percentage of total N of protein	2·48	2·89	2·77
'Sullivan N' of unhydrolysed 'basic peptide fraction' in percentage of total N of protein	0·22	0·00	—
A	2·26	2·89	2·77
B	6·9	9·3	7·2
'Ninhydrin-CO_2' value for unfractionated partial hydrolysate (cf. Table 2) as N in percentage of total N of protein	26·4	23·4	33·7
C	24·1	20·5	30·9
'Ninhydrin-CO_2' value for unfractionated complete hydrolysate (cf. Table 2) as N in percentage of total N of protein	68·6	63·4	81·5
D	61·7	54·1	74·3
A/B	0·33	0·31	0·38
C/D	0·39	0·38	0·41

The ninhydrin-CO_2 value (same units as A) for the unfractionated partial hydrolysate of the protein (cf. Table 2) *minus* A, gives the free neutral and acidic amino-acids in the partial hydrolysate (C), while the ninhydrin-CO_2 value for the complete hydrolysate of the protein (cf. Table 2) *minus* B, gives the total neutral and acidic amino-acid residues of the protein (D). The desired ratios are then expressed as A/B and C/D.

It is seen that while a consistently lower proportion of the basic amino-acids is set free than of the neutral and acidic amino-acids, the difference between the two categories is not a difference of order, and does not justify Siegfried's original claim. An increased resistance of basic amino-acid residues in peptide linkage to acid hydrolysis is anyway to be expected, owing to the repellent effect on the H^+ ions of the positively charged group in the side-chain, though the effect of this is not likely, owing to its greater distance from the bond to be split, to be comparable with that of the $—NH_3^+$ or $=NH_2^+$ group of a simple peptide.

Cystine of the basic peptides

The 'basic peptide fraction' from wool gave a Sullivan value before it was hydrolysed with acid. This increased after hydrolysis, as shown in Table 10. It is not necessary, in order to explain the value before hydrolysis, to postulate that cysteine residues (½-cystine residues) involved in a peptide bond react, since free ½-cystine residues linked to a bound ½-cystine residue through a disulphide bridge would be expected to give a Sullivan reaction:



The results obtained are of some interest as throwing light on the mode of incorporation of cystine residues in wool.

In the case of edestin, the isolation of cystine peptides would be of special interest in view of the fact that it liberates —SH groups on denaturation [cf. Greenstein, 1939] and in view of Linderstrøm-Lang & Jacobsen's [1941] interesting 'thiazoline' hypothesis in explanation of this phenomenon. Unfortunately with this protein all the ½-cystine residues are set free in the course of partial acid hydrolysis, and none appear in the 'basic peptide fraction'.

Serine and threonine of the basic peptides

That these residues in partial acid hydrolysates of the proteins investigated have their amino-groups liberated with especial readiness has been mentioned in § I. This is borne out for the 'basic peptide fractions' by determination of 'total hydroxyamino-acid' before and after hydrolysis (cf. Table 10). A decrease of this value was observed on hydrolysis, suggesting partial destruction of these residues during the hydrolysis of seryl and/or threonyl peptides. Some of the ammonia formed during this treatment may have its origin in these compounds (cf. Table 11).

Owing to the probable presence of a considerable proportion of hydroxylysine residues in the 'basic peptide fraction' obtained from gelatin, no attempt has been made to evaluate the neutral hydroxyamino-acids present in this fraction.

Neutral amino-acids of the basic peptides

Subject to the reservation mentioned in the introduction to this paper, that the peptides studied may conceivably be artefacts arising by rearrangement, the results of the present work are of some interest in considering the arrangement of amino-acid residues in proteins. Nearly all the neutral amino-acids found in a hydrolysate of the 'basic peptide fraction' derived from the proteins studied may be inferred to have been directly linked to a basic amino-acid, since the basic peptides present have been shown to be almost all dipeptides. No neutral amino-acid residue present in the original protein has been found to be absent, on searching for it, from the 'basic peptide fraction' except cystine, in the case of edestin, which has been shown to be set free completely during the hydrolysis. The control experiments already described make it extremely unlikely that neutral residues can enter this fraction in the form of free amino-acids. Nor are the proportions in which the various neutral amino-acid residues occur in the 'basic peptide fraction' very different from those in which they occur in the original protein. This is illustrated in Tables 15 and 16. In Table 16

Table 15. *Estimate of 'neutral amino-acid' content of proteins and derived basic peptide fractions*

	Wool		Edestin		Gelatin	
N as % of total N of protein or fraction	Whole protein	'Basic peptide fraction'	Whole protein	'Basic peptide fraction'	Whole protein	'Basic peptide fraction'
'True amide-N'	7·2	0·0	9·4	0·0	0·5	0·0
Arginine-N	20·2*	59·7	28·1†	70·4	14·6‡	53·2
Lysine-N	3·1§	9·2‖	2·4†	6·1‖	6·3‡	22·9‖
Histidine-N	1·2§	3·5‖	3·4†	8·5‖	1·3‡	4·7‖
Glutamic acid	6·0¶	—	10·4†	—	3·1‡	—
Aspartic acid	3·6¶	—	6·8†	—	2·0‡	—
Total of above	41·3	72·4	60·5	85·0	27·8	80·8
N of neutral amino-acids (by difference)	58·7	27·6	39·5	15·0	72·2	19·2

* Vickery [1940].
† Private communication from Prof. A. C. Chibnall.
‡ Dakin [1920].
§ Block [1939].
‖ Estimated.
¶ Private communication from Dr K. Bailey (minimum figure).

Table 16. *N of individual neutral amino-acids as % of total 'neutral amino-acid-N' of protein or derived fraction* (cf. Table 15)

	Wool		Edestin		Gelatin	
Determination*	Whole protein	'Basic peptide fraction'	Whole protein	'Basic peptide fraction'	Whole protein	'Basic peptide fraction'
Cystine-N	13·4	10·5	0·9	0·2	—	—
Tyrosine-N	3·6	6·0	3·3	7·0	—	—
Hydroxyamino-acid-N	20·8	19·2	17·7	15·7	†	†
'Higher monoamino-acid-N'	26·0	38·4	31·9	67·0	12·8	30·2
'Proline-N'	12·0	15·9	10·4	12·7	15·8	21·8

* The original determinations are shown in Tables 11 and 12. The figures taken from Table 11 are, where possible, the mean of two determinations.
† Not calculated, because of probable presence of hydroxylysine.

the N of the individual neutral amino-acid or group of neutral amino-acids is expressed as percentages of the total N of the neutral amino-acid residues present in the 'basic peptide fraction' and in the parent protein respectively.

This 'total N of neutral amino-acids' is arrived at (see Table 15) by subtracting from the total N of the protein, the N of arginine, histidine and lysine, the 'true amide-N' (cf. § I) and the N of glutamic and aspartic acids. For the 'basic peptide fractions' glutamic acid, aspartic acid and 'amide-N' are deemed absent. The lysine and histidine contents of the 'basic peptide fractions' have not been determined, but they are estimated as being present in the same ratio to the arginine as in the parent protein. This assumption introduces a source of second-order error only, and does not affect significantly the general conclusion.

It is not easy to reconcile the picture of protein structure presented by the present work with the Bergmann-Niemann hypothesis, according to which in general the residues of any particular amino-acid will be directly linked to only a small number of different amino-acid species. Thus for silk [cf. Bergmann & Niemann, 1938], given the structure

$$—G—A—G—R—G—A—G—R—G—A—G—R,$$

where

$$\left.\begin{array}{l} G = \text{glycine residue} \\ A = \text{alanine residue} \\ R = \text{other residues} \end{array}\right\} \text{ in peptide chain,}$$

a pentapeptide is the smallest molecule that can incorporate a residue of a basic amino-acid together with a residue of any amino-acid other than glycine or alanine. Similarly for gelatin [cf. Astbury, 1940]:

$$—P—G—R—P—G—R—P—G—R—,$$

where

$$\left.\begin{array}{l} G = \text{glycine residue} \\ P = \text{proline or hydroxyproline residue} \\ R = \text{other residues} \end{array}\right\} \text{ in peptide chain,}$$

a tetrapeptide is the smallest molecule that could contain a basic residue together with one of any species other than glycine, proline or hydroxyproline, unless the residue replacing one out of every 18 proline-hydroxyproline residues is involved.

While the present work does not by any means form a rigid refutation of the Bergmann-Niemann hypothesis, it seems to suggest for proteins a considerably more complicated structure. The hypothesis of random structure, with which it is scarcely incompatible, may be rejected on physical and biological grounds.

At present, the most hopeful line of extension of the work seems to be towards the isolation from a physically well-characterized and homogeneous protein of peptides containing a high proportion of the total residues present of one particular amino-acid. The smaller the number of such residues in the molecule the simpler should be the peptide mixture to be studied, and in the limiting case of one residue of the amino-acid in question per molecule, only two dipeptides should be obtainable containing this residue on any hypothesis of regular structure based on the peptide theory. Such a study, as mentioned above, would also be a valuable control on artefacts arising by rearrangement. Work in this direction is at present in progress.

SUMMARY

1. The characterization of the lower peptides resulting from the partial hydrolysis of proteins, and its significance for the elucidation of protein structure are discussed.

2. Concentrated hydrochloric acid, acting on wool, edestin and gelatin at 37° for a week, liberates about a third of the residues as free amino-acids. The rest are present in the form of lower peptides.

3. The general kinetics of this acid hydrolysis are described, with special reference to the splitting of bonds involving cystine, serine, threonine, proline, hydroxyproline and ammonia residues.

4. By electrodialysis in a diaphragm cell it has been found possible to isolate a large proportion of the basic amino-acid residues in the form of dipeptides.

5. A study has been made of the amino-acids directly linked to the basic residues. Its bearing on past and current theories of protein structure is discussed.

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153. SURFACE CHEMISTRY OF CAROTENOIDS

I. ASTACENE[1]

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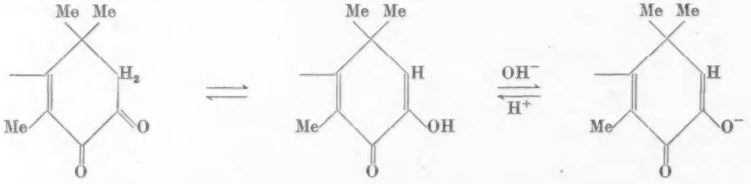
Astacene, a carotenoid derived from the naturally occurring pigment astaxanthin [Kuhn & Sorensen, 1938] commonly found in many crustaceans, is a tetraketo-β-carotene [Karrer & Loewe, 1934; Karrer *et al.* 1935] bearing two keto groups on each of the closed ionone rings which terminate a long polyene chain. Tautomeric keto-enol equilibrium conditions render astacene capable of acting as a weak acid, showing reversible formation of salts in the presence of alkali:



The ready hydrolysis of K salts of such carotenoid acids as astacene and metridene, an astacene-like carotenoid from the anemone *Metridium* [Fox & Pantin, 1941], is shared by weak carboxylic acids such as the indicator methyl red. Unless considerable excess base is present in the aqueous phase such salts hydrolyse at the interface between light petroleum and water, and diffuse as the corresponding free acids into the light petroleum layer.

Astacene and metridene are yellow in light petroleum solution but orange-red in pyridine, magenta-red in CS_2 and various yellow, orange or red colours in other organic solvents. Either of these carotenoids, when their light petroleum solutions are shaken vigorously with water, form decidedly pink evanescent oil-in-water emulsions. As these emulsions rapidly break, the pigment diffuses back into the light petroleum layer, again yellow in colour. This reversal of the colour-change with the breaking of the emulsion clearly indicates a difference between the absorption spectra of molecules adsorbed at the light petroleum-water interface and those in bulk light petroleum solution.

The colour-change of astacene may be due to any of several factors:

(1) The effective solvent of astacene at the light petroleum-water interface may differ from pure light petroleum solution. For example, its effective dielectric constant must lie between that of water and that of light petroleum.

[1] Spectroscopic observations by D. L. F.; monolayer measurements by J. F. D.; chromatic emulsions studied jointly.

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Thus the different colour of the interfacial astacene molecules may be due to their being in a different solvent.

(2) If enolic derivatives of astacene are red, such a colour would be produced if the keto-enol equilibrium were shifted in the interface toward the enol form.

(3) If the ion of astacene is red, an increase in ionization of astacene in the interface might be responsible for the shift in colour from yellow to red.

Experimental

Astacene, derived from the astaxanthin of lobster eggs, was recrystallized from pyridine. Surface pressure and surface potential measurements of monolayers were made with techniques described by Adam [1938]. Determinations of absorption maxima were made with the use of a recent model of the Hartridge Reversion Spectroscope [cf. Emerson & Fox, 1940; Fox & Pantin, 1941]. Glass-distilled water was used in all the experiments.

Results: I. With emulsions. Solutions of 20 mg. astacene per l. of light petroleum were shaken with various aqueous solutions. With water, 5*N* HCl, *N*/10 HCl, 5*N* KOH, 5*N* NaOH, *N*/10 NaOH and saturated NaCl solution, the change in colour to pink on shaking was found. Deutsch [1927] has found that colour changes due to ionization of weak acids at interfaces are a function of the *p*H of the aqueous phase. This is not the case with the colour change of astacene, although astacene is a very weak acid. Hence the pink colour cannot be due to ionization; i.e. explanation (3) may be discarded.

When astacene in light petroleum was shaken with 50 % acetic acid, 50 % aqueous pyridine, 50 % methyl or ethyl alcohol, 50 % acetone, 1 % octyl alcohol, 1 % butyric acid or water containing traces of oleic or palmitic acids, the colour change from yellow to pink no longer occurred. While the presence of CS_2 in the light petroleum did not affect the phenomenon, addition of $CHCl_3$ or diethyl ether inhibited it considerably. It also was abolished if traces of cholesterol, lecithin or triolein were added to the light petroleum before shaking with water. The colour change did not vanish abruptly at a particular concentration of an inhibiting substance, but over a range of concentrations of 10-fold to 100-fold. Thus with acetic acid the inhibition of change occurred over the concentration range of 1 % to 10 % in the aqueous phase, with pyridine, between 1 % and 0·01 %, in the aqueous phase; with oleic acid, between 0·1 % and 0·001 %, in the light petroleum phase.

On the other hand, when shaken with Na butyrate, Na oleate, or Na palmitate solution, the pink colour was not suppressed, but possibly even accentuated, and a permanent emulsion formed. The colour change with these soaps appeared only at *p*H values more alkaline than about 11·5. At *p*H 12, a concentration of Na oleate in the aqueous phase 10^4 times greater than the astacene concentration in the light petroleum did not cause inhibition of the colour change. We may provisionally suggest that the persistence of the colour change with soaps is due to formation of a complex between astacene and fatty acid anion; and that, since there is no colour change at *p*H 9 where ionization of fatty acid is almost complete, whereas the change is pronounced at *p*H 12 where the anion of the enol form of astacene probably begins to appear, the complex is possibly formed between fatty acid anion and astacene anion, and not with unionized astacene. This, however, has not been proved.

Summing up the results obtained with emulsions, we may say that all surface-active materials tried, with the exception of soaps, suppress the colour

change. This may reasonably be due to competition of molecules for a position in the petrol-water interface, so that in the presence of sufficient amounts of other surface-active material, astacene molecules are unable to secure a position in the interface, and consequently no colour change occurs. The apparent exception of soaps may be due to formation of a complex, so that in this case astacene molecules are not driven out of the surface; the evidence for the existence of such a complex will be discussed later.

Results: II. Absorption bands in different solvents. It is well known that the absorption bands of carotenoids in the visible region of the spectrum vary from solvent to solvent. Table 1 shows values for the position of the maximum

Table 1. *Position of the absorption band of astacene in the yellow-red zone of the visible spectrum*

Substance	mμ	Colour of solution
Light petroleum	483	Yellow
*cyclo*Hexane	489	Yellow-orange
Acetone	489	Orange
Glacial acetic acid	488	Orange
Benzene	496	Red-orange
Chloroform	497·5	Orange-red
Pyridine	500	Orange-red
Carbon disulphide	512	Red

of the adsorption band of astacene in the region of 490 mμ in various solvents. The changes in colour may involve enolization, but since a similar change is found with other carotenoids which have no polar groups at all, such as β-carotene, it is more probable that the main action of the solvent is on the polyene chain. Table 2 shows the shift in the position of the absorption band

Table 2. *Shift in absorption band on emulsification of light petroleum solutions of carotenoids with aqueous K oleate*

Compound	Absorption band of light petroleum solution: (*a*) Without oleic acid mμ	Absorption band of light petroleum solution: (*b*) With oleic acid mμ	Absorption band of light petroleum solution emulsified with K oleate mμ
β-Carotene	481	481·5	485*
Zeaxanthin (dihydroxy-β-carotene)	480	482	489·5*
Astacene (tetra-keto-β-carotene)	483	484	493

* First absorption band very diffuse in presence of soap, and difficult to determine accurately; second absorption band, recurring at about 451 mμ obliterated (in Hartridge instrument) in presence of soap.

when a solution of a carotenoid is emulsified in water containing a little K oleate. β-Carotene shows a shift of 3·5 mμ, zeaxanthin of 7·5 mμ, and astacene of 9 mμ. The shifts with the former two substances are not visible to the naked eye, which is probably the main reason why such shifts have not been previously reported.

Thus the shift of absorption band is not confined to astacene, but is also exhibited, though to a smaller degree, by other carotenoids. The values of the shift have the order: carotene < zeaxanthin < astacene. Since these substances have respectively 0, 2 and 4 polar groups, it is to be expected that their tendency to accumulate at an air-water interface will follow the same order, carotene < zeaxanthin < astacene. Hence, if the shift on emulsification is due to the difference between the properties of the interface and bulk phases, it is possible that

the differences in the amount of shift are due simply to the fact that the amounts of the substance actually in the interface follow the series carotene < zeaxanthin < astacene: i.e. the greater shift found with astacene may simply be due to a larger fraction of astacene entering the surface phase on emulsification than is the case with the other substances. Consequently it is not essential, on the evidence at present available, to attribute the larger shift with astacene to enolization. On the other hand, the possibility of enolization being involved cannot be ruled out.

Results: III. With monolayers. Fig. 1 shows surface pressure and surface potential (ΔV) measurements on astacene monolayers spread on 0·01 N HCl. The colour of the monolayers was obvious even at an area of 100 Å.^{2} per molecule.

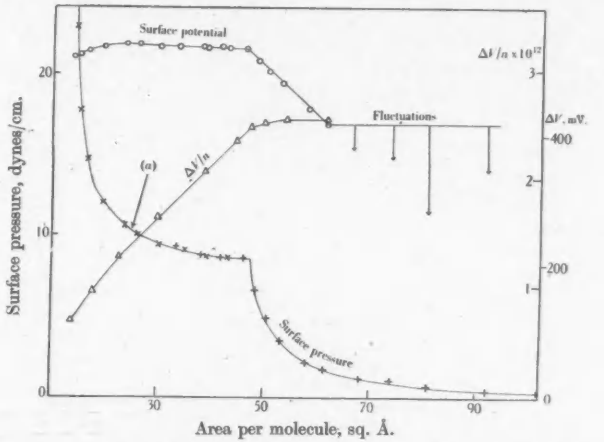

Fig. 1. Surface pressure and surface potential measurements on monolayers of astacene, spread on 0·01 N HCl. The film is solid at areas less than that indicated by (*a*).

At areas greater than 61 ± 2 Å.^{2} the film is electrically heterogeneous, ΔV showing large fluctuations. On compression from 61 Å.^{2} to 47 Å.^{2} the surface pressure rises to 8·9 dynes/cm., the film being electrically homogeneous over this range, ΔV rising from 420 to 530 mV. At 47 ± 1 Å.^{2} there is a discontinuity in both surface pressure and ΔV curves. On compression to areas smaller than 47 Å.^{2} the surface pressure rises slowly, until the film solidifies at 25 ± 1 Å.^{2}, and ΔV rises slowly from 530 to 545 mV. At areas less than 25 Å.^{2} the film is much less compressible, and tends to collapse. Measurements taken rapidly at higher pressures when extrapolated to zero pressure suggest a limiting area of about 18 Å.^{2} for this incompressible part of the film, but this value may be in error, due to collapse. The curve of $\Delta V/n$,* where n is the number of molecules per cm.^{2}, also shows a transition at 47 Å.^{2} Between 47 and 61 Å.^{2}, $\Delta V/n$ is roughly constant. At areas less than 47 Å.^{2}, $\Delta V/n$ falls rapidly as the area per molecule is diminished.

The general behaviour of the films is similar to that of films of other molecules containing two sets of polar groups widely separated by a hydrocarbon chain [Adam & Jessop, 1926; Adam *et al.* 1934] or by a hydrocarbon ring system

* $\Delta V/n$ is a measure of the average contribution per molecule to the observed surface potential.

[Adam *et al.* 1932; Danielli *et al.* 1933]. It therefore seems probable that the characteristics of astacene films are to be explained in the same way as those of, for example, the dibasic esters $CO_2Et.(CH_2)_n.CO_2Et$, or triacetyl oestrin. Thus at areas greater than 47 Å.^{2} per molecule all the polar groups of astacene must be in the water surface, and the transition region between 47 and 25 Å.^{2} is the region in which one set of polar groups of each molecule is removed from the surface, so that at areas less than 25 Å.^{2} the molecules are all standing upright, with one polar group only in the surface, giving an incompressible film. At 47 Å.^{2} the astacene molecule must be bent in a bow, for the area of 47 Å.^{2} is sufficient only for the rings containing the polar groups, and the hydrocarbon chain linking the two rings must be raised above the water surface. This is evidence for considerable flexibility of the polyene chain. The areas of 47 Å.^{2} for two rings in the surface, and of 25 Å.^{2} for the solid film with one ring in the surface, are similar to those given by Adam for simple benzene ring systems. The apparent limiting area of 18 Å.^{2} at zero compression is low for a ring system; as mentioned before, this area may be in error due to collapse.

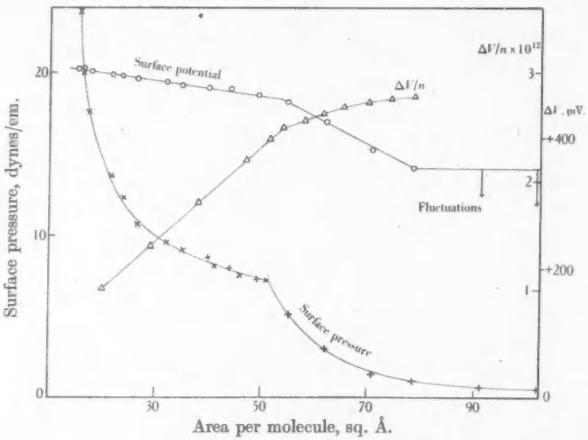

Fig. 2. Surface pressure and surface potential measurements on monolayers of astacene, spread on $N/6$ NaOH.

Fig. 2 shows results for astacene films spread on $N/6$ NaOH. The films are similar in type to those on $N/100$ HCl, but occupy rather larger areas per molecule at a given surface pressure, and ΔV is about 50 mV. lower than on HCl solution. The transition point on the surface pressure, ΔV, and $\Delta V/n$ curves is at 53 ± 2 Å.^{2}

Films were also spread from solutions containing equal numbers of molecules of oleic acid and astacene. Molecules in such films spread on $N/100$ HCl behave in the same way as in films of the separate molecular species, i.e. if it is assumed that in the mixed film oleic acid occupies the same area, and gives the same surface potential at a given surface pressure, as it does in films containing oleic acid only, then the areas and surface potentials calculated for astacene in the mixed film are also the same as in films containing astacene only. The only exception to this is the surface potential, which is greater for astacene in the mixed film than in the film containing astacene only by 70 mV. at an area of

25 Å.² The difference falls to zero at 47 Å.², within experimental error. This suggests a slight interaction between the dipoles of oleic acid and astacene, occurring only when the latter molecules are standing on end with only one ring system in the interface.

Fig. 3 shows results for oleic acid on $N/6$ NaOH; A for films consisting of oleic acid only; B values calculated for oleic acid from results obtained on films containing equal numbers of molecules of oleic acid and astacene, assuming that in the mixed films astacene occupies an area and gives a surface potential, at a given surface pressure, equal to that given by astacene in films containing astacene only. Oleic acid alone forms a film of gaseous type, with a constant surface potential of −60 mV. In the mixed film, at surface pressures of less

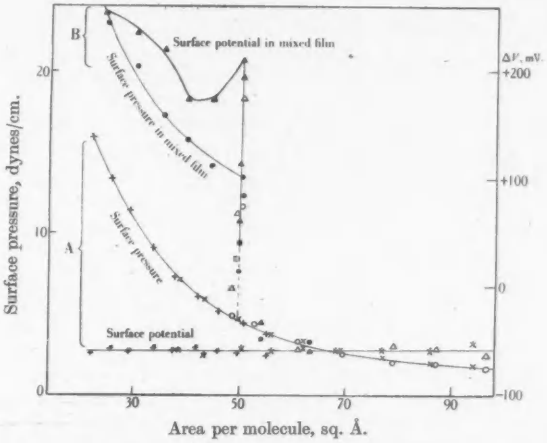

Fig. 3. Surface pressure and surface potential measurements for monolayers of oleic acid spread upon $N/6$ NaOH. Curves A: for films consisting of oleic acid only. Curves B: results calculated for oleic acid from observations made on films containing equal numbers of molecules of oleic acid and of astacene, assuming that the behaviour of astacene molecules on the surface is not changed by the presence of oleic acid molecules.

than 4·5 dynes, the calculated area and surface potential for oleic acid are the same as is found for oleic acid alone. But as the pressure is raised from 4·5 dynes to 13·5 dynes, the calculated area per molecule for oleic acid remains nearly constant, and the surface potential rises from −60 to +210 mV. This range of surface pressures is that in which one ring system of each astacene molecule, with its polar groups, is removed from the interface: at the surface pressure of 4·5 dynes, where this change begins, astacene occupies 51 Å.² On further compression of the mixed film the area per molecule calculated for oleic acid falls again, from 50 Å.² at 13·5 dynes to 24 Å.² at 24 dynes, above which surface pressure the mixed film tends to collapse.

From these results the following conclusions may be drawn concerning the mixed film on $N/6$ NaOH: (1) at areas at which astacene has both rings in the interface, there is very little interaction between the oleate ion and astacene; (2) there is a very strong interaction between the oleate ion and astacene molecules oriented with one ring only in the interface and having the polyene chain

roughly perpendicular to the surface. This interaction involves a surface potential increase of +270 mV. and an area increase of 25 Å.^{2} per molecule at a surface pressure of 13·5 dynes, at which the interaction appears to be most marked. It is probable, though not known with certainty, that on $N/6$ NaOH astacene is ionized, so that the interaction is probably between oleate ion and astacene ion, and not between oleate ion and unionized astacene.

This interaction is probably similar to those recently studied by Schulman & Rideal [1937]. Schulman & Stenhagen [1938] and Schulman & Cockbain [1938; 1939] have shown that where a large interaction takes place between two molecular species in an interface, a mixed film of the two species is often more stable than a film of either species alone, and that an initially formed film of one species, when the second species is added to the system, may be converted into the more stable mixed film. The interaction between oleate ion and astacene is very large, and hence the astacene-oleate ion mixed film would be stable at an interface on this view even in the presence of an excess of oleate ion. But the interaction between free oleic acid and astacene is slight and accordingly there is no reason to suppose that a mixed film will be more stable than a film of oleic acid, in the presence of an excess of oleic acid. This may be the reason why free oleic acid can drive astacene out of the oil-water interface, whereas oleate ion, at alkaline *p*H, cannot do so.

Discussion

Of the three possible factors which might cause the absorption spectrum of astacene at an interface between water and light petroleum to be different from the absorption spectrum of astacene in a bulk phase of light petroleum, the third possibility (increased ionization at the interface) has been eliminated. The first factor, that the interface is a different solvent, is probably the major one, since it has been shown that other carotenoids which neither ionize nor enolize show a similar change following absorption. The shift is such that the interface behaves as though it were a more polar solvent than light petroleum but less polar than $CHCl_3$. Adam and Rideal and their colleagues have advanced the view that molecules in monolayers of fatty substances at a water surface will behave as though they were in a solvent of properties intermediate between those of water and a pure hydrocarbon liquid. The position of the absorption bands of carotenoids in a monolayer is consistent with this view.

But although the character of the interface as a solvent is sufficient to account for the shift in absorption spectrum of astacene, the evidence at present available is not sufficient to exclude enolization as a contributing factor.

Summary

Astacene in light petroleum is yellow: when the solution is emulsified with water the colour changes to pink. This change is not affected to any marked degree by *p*H or salt concentration. It is suppressed by other surface active substances, with the exception of the anions of fatty acids.

The position of the absorption band of astacene in the yellow-red region of the spectrum has been measured in different solvents. In more polar solvents the band is further towards the red region. The position of this band, in light petroleum solution and in emulsified light petroleum solution, has been measured for astacene, β-carotene and zeaxanthin. In all cases the band is shifted to longer wave-lengths by emulsification, the effect being least with carotene, greatest with astacene.

Surface pressure and surface potential measurements have been made on monolayers of astacene, and of astacene mixed with oleic acid. On acid solution there is little interaction in the monolayers between the two sets of molecules: on alkaline solution there is a pronounced interaction.

The cause of the colour change on emulsification is discussed. It is attributed mainly to the more polar character of the interface compared with light petroleum, but in the case of astacene enolization may also be involved.

We are indebted to Dr Kidd for his kind assistance in obtaining a supply of lobster eggs; to Mr McBey for obtaining eggs under conditions of some difficulty; to Mr J. L. Parkinson for construction of the surface potential apparatus; to Mr G. W. Spicer for preparing polonium electrodes; and to Dr J. H. Schulman for reading the manuscript.

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154. THE ASCORBIC ACID CONTENT OF DRIED VEGETABLES

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In Kenya there is a multitude of climates, from humid tropics to eternal snow, from rain forests to desert. The problem of providing an adequate supply of fresh vegetables might thus appear simple, but the distance dividing the cool temperate zones, suitable for vegetable production, and the semi-arid to desert zones is great. The transport of fresh vegetables is not always easy and wastage is heavy. The use of dried vegetables seemed worth exploring; they are easy to transport and, if properly prepared, palatable. The problem was placed before us and we were asked to take into consideration two criteria—palatability and vitamin C content. There was little information of previous work at our disposal so the problem had to be undertaken as a subject of initial research employing the knowledge gained in pyrethrum drying.

When the problem was first propounded we were interested only in rations for the native population. One of the most popular of the local green vegetables being the young leaves of the cowpea (*Vigna catjang*), this material was used in our preliminary experiments. The leaves were dried in the sun and by artificial methods. The results were not too encouraging. (For method of estimating ascorbic acid see below.)

Table 1. *Ascorbic acid content of fresh and dried cowpea leaves*

	mg./100 g.	Calculated to fresh weight
Fresh leaves	13·2	—
Sun-dried	15·6	2·5
Artificially dried	33·8	5·5

Although these figures are not strictly comparable they were sufficient to decide us against attempting to produce dried vegetables by sun-drying only. A further experiment was made in a drier. Fresh leaves containing 70 mg. ascorbic acid per 100 g. gave 16 % of dry material, containing 237 mg. ascorbic acid per 100 g., a loss of 46 %: apparently artificially dried cowpea leaves would be a good antiscorbutic. There remained the other test, that of palatability; the dried cowpea leaves were cooked at the labour camp at these laboratories in the normal native manner. The report was that the material was quite edible but very hard and fibrous. Apparently, during drying, the vascular bundles undergo an irreversible change and are no longer capable of absorbing water and becoming soft. This is not an insuperable difficulty but is certainly a grave disadvantage.

Experiments were now extended to kale, not because it was a likely vegetable but because a supply was readily available and it is a type of crucifer. The experiments were really intended to ascertain the most suitable temperature for artificial drying. Results of the first series are given in Table 2.

Table 2. *Effect of temperature and time of drying on kale*

Temp.	45–48°	53–55°	60–65°
Ascorbic acid in fresh, mg./100 g.	173	147·5	181
Loss of weight in drying, %	79·3	83·5	79·5
Time of drying, hr.	9	4	3
Ascorbic acid in dry, mg./100 g.	295	196	170
Loss in ascorbic acid, %	65	77·5	81

In this series of experiments the rates of loading in the drier employed were practically identical. Although drying at a lower temperature is protracted, the oxidation of ascorbic acid is much less than at the higher temperatures. Other experiments on the same material gave comparable results, but more important was the indication that, in such experiments, uniformity of technique is essential. In the second series of experiments the destruction of ascorbic acid was over 90 %, this great loss being attributable to bruising during preparation.

Further work was done on lucerne. Dried lucerne was found to contain a large amount of ascorbic acid [Levy & Fox, 1935] but here too the dehydration of the vascular bundles gave a very fibrous cooked material. Although these preliminary experiments were conducted upon what appeared to be quite suitable and very easily obtained vegetables, there were several reasons why others should be examined. The main objection against cowpea leaves was the development of the hard fibre, which definitely reduced palatability. Work was not continued on lucerne because of observations made in South Africa that lucerne was liable to cause digestive disturbances. Kale was omitted because of the difficulty in handling it.

Most, if not all, European vegetables can be grown in the highlands of Kenya. Most of these are suitable for drying, but there are reasons for not using them all. For example, asparagus grows to perfection but it takes time to grow and is always a luxury product.

Experimental

The type of drier employed in the early stages of these experiments was a cylindrical electrically heated one provided with thermostatic control, having a single tray of 1 sq. ft. area; the draught was comparatively low, being supplied by a chimney 4 ft. high. Distribution of heat in this drier is rather uneven, and the material being dried requires constant attention. Later, a model drier of the type employed for pyrethrum drying [Beckley & McNaughtan, 1937] was built. This drier is square with 3 ft. sides and carries 6 trays. Heating is electrical, thermostatically controlled. Ventilation is secured by means of a 6 in. pipe, 8 ft. long; later this was augmented by a small electric fan. On the whole, heat distribution in this drier is fairly regular but on days of high wind, fluctuations in the distribution of heat occur, although the drier is indoors.

In the course of experiments conducted on the full scale, it was decided to change to a tunnel drier. Logically, therefore, laboratory scale experiments should also have been made on a tunnel drier. Previous investigations on a different subject, however, demonstrated that it is very difficult to build an efficient model tunnel type drier. However, the experience gained in full scale experiments, to be reported later, showed that the effects obtained in the model drier used by us are not very different from those obtained in a full-size tunnel drier.

Ascorbic acid was assayed by titration with indophenol-2:6-dichlorophenol solution. A 3 % solution of metaphosphoric acid was employed in grinding and,

where liquids were concerned, such as the water obtained in cooking the vegetables, the final concentration was made up to 3 % by the addition of the requisite quantity of 20 % metaphosphoric acid.

Cabbage. The preparation of cabbage for dehydration consists in cutting it into shreds $\frac{1}{4}$ to $\frac{1}{2}$ in. in width. In view of the effect of metals upon the ascorbic acid oxidase an experiment was conducted to ascertain the effect of using ordinary steel and stainless steel knives for shredding, upon the ascorbic acid content of dried material. A small cabbage was halved using a stainless steel knife and the core carefully removed. One-half was shredded with a stainless steel knife, the other with an ordinary knife, and equal quantities of the shredded material immediately transferred to the drier. A single tray divided along the middle was used for both lots. In order to ensure that each half received similar treatment the tray was turned at intervals. The moisture contents of the two lots, 6·08 % and 6·11 %, show that drying in the two halves was very even. The dried cabbage shredded with a stainless steel knife had an ascorbic acid content of 478 mg./100 g.; that shredded with an ordinary knife assayed at 302 mg./100 g. Because of this big difference stainless steel knives were employed throughout the subsequent work.

Normally, cabbages come from the field with a fair number of outer green leaves. Some of these are very coarse and not suitable for food. In addition, the outer leaves are not easily shredded by hand, for which reason in our very early experiments the firm heads only were used. This gave a great deal of waste; when in one case the waste reached 70 % of the original weight, we were led to investigate the distribution of ascorbic acid in a head of cabbage. Good firm cabbages were taken and only the very coarse outer green leaves were discarded. The outer leaves contained by far the highest relative proportion of ascorbic acid, followed by the core and the small heart leaves. The tender white inner leaves contained very much less. This observation is confirmed by Robinson & Courtice [1940]. The results of three experiments are given in Table 3.

Table 3. *Distribution of ascorbic acid in the cabbage, mg./100 g.*

Outer green leaves	85·7	63·0	104·6
Curled green leaves	68·9	49·4	55·2
White leaves of head	45·2	46·0	32·5
Heart leaves	78·0	65·7	55·0
Core	77·9	66·3	56·7

As a result of these experiments all the outer leaves which appear to be sufficiently tender are now to be included in the material to be shredded and dried. On the large scale the shredding machine will do this easily.

From information which reached us it appears that in preparing cabbage for drying it is usual to blanch the shredded material in live steam for a couple of minutes. Such treatment by killing the tissues speeds up drying. It is also claimed—quite probably correctly—that this preliminary steaming destroys the oxidases and so reduces the loss of ascorbic acid.

The results of one of our experiments to ascertain how blanched dried material compares with unblanched dried material are given in Table 4.

The dried blanched material contains about 20 % more ascorbic acid than does the dried unblanched material. In cooking too there is a marked difference. The cooking water from the dried unblanched material contains 19 % of the original content, while the solid contains only 12·6 %, a loss during cooking of 68·4 % of the ascorbic acid in the original material. The cooking water of the blanched material contained 28·9 % and the drained solids 16·8 % of the original

Table 4. *Effect of blanching on ascorbic acid content of dried cabbage, mg./100 g.*

Fresh cabbage	63·0
Unblanched dried, boiled 30 min. after soaking	332·5
Cooking water	63·3
Cooked solid	41·8
Blanched dried, boiled 30 min. after soaking	406·7
Cooking water	117·4
Cooked solid	68·4

content. The loss in cooking in this case is only 54·3%. A further advantage is that the cooked solid from the blanched material contains half as much again ascorbic acid as that from the unblanched material. As far as ascorbic acid is concerned blanching is definitely desirable. The ascorbic acid content is not the sole criterion; palatability of the final product must also be taken into account. On soaking, blanched material absorbed six times its weight of water, unblanched material four times. The soaked material was then cooked for 30 min. Perhaps the time of cooking may appear excessive but all these experiments have been conducted at an altitude of 5800 ft., where cooking takes longer than at sea level. Throughout the cooking the material was tested at intervals with the point of a glass rod. Partially cooked blanched material at 20 min. looked very attractive, but after a full 30 min. a very marked change in appearance had occurred; the green remained, but looked artificial, the white portions assumed a greyish tint while the whole was reduced to a mere mush, unattractive in appearance and most unpalatable. The unblanched material under exactly the same conditions was crisp, most attractive in appearance and indistinguishable from well cooked fresh cabbage.

The very great loss of ascorbic acid during the soaking and cooking of the unblanched dried material is a very grave disadvantage. In such material of course the oxidases are not destroyed and during the period of soaking would act upon the ascorbic acid. That this is so is demonstrated by the results of experiments, one of which is given in Table 5, in which soaking followed by boiling was compared with dropping the material directly into vigorously boiling water. In another experiment the loss after soaking and cooking was 64·5%, after boiling only 39·6%. There is no doubt that immediate boiling has prevented the destruction of ascorbic acid.

Table 5. *Effect of soaking before boiling on ascorbic acid content of dried unblanched cabbage, mg./100 g.*

Dried cabbage, boiled only	434·2
Cooking water	186·3
Cooked solid	176·8
Loss in cooking	16·4%
Soaked and boiled:	
Cooking water	83·3
Cooked solid	52·0
Loss in cooking	68·9%

As regards palatability there was no difference either in appearance or in taste between the two lots of cabbage. With cabbage, therefore, there is no need for preliminary soaking.

It would appear that the preparation of cabbage for dehydration is a simple matter. In a full scale experiment where a ton of cabbage was treated, the final product contained 194 mg. ascorbic acid/100 g., quite a satisfactory figure considering that the material was cut up by inexperienced labour. Material produced

by a local farmer, who was very keen to embark upon the drying of vegetables, contained only 35 mg./100 g. There are two possible explanations of this very low content. Blunt knives may have been used causing bruising, or the shredded material may have been stored for some time before being placed in the drier. That this is a serious matter is shown by the following figures: some shredded material which contained 55·4 mg./100 g. contained only 30·8 mg./100 g. after being left for 5 hr., a loss of 45 % of the ascorbic acid content.

For the full scale production special machinery is being ordered in which the cutters are all stainless steel, and very great care will be taken that cabbage in the shredded condition will not stand more than a few minutes before going into the drier.

To obviate criticism that palatability trials had been confined to members of the laboratory staff fair-sized samples were sent to a number of people living at a distance. In every case they reported that the material was excellent and most palatable. In one case the sample was 3 months on the road and subjected to all sorts of climatic conditions.

Cauliflower. In view of the unexpected distribution of ascorbic acid in the cabbage a similar study was made on the cauliflower. Again the highest content was found in the outer leaves and in the innermost stalks. The results of an experiment are given in Table 6.

Table 6. *Distribution of ascorbic acid in the cauliflower, mg./100 g.*

Outer leaves	83·9
Outer 'flowers'	65·2
Stalks of outer 'flowers'	63·8
Inner 'flowers'	48·0
Stalks of inner 'flowers'	70·5

Since normally the outer leaves of a cauliflower are more tender than the outer leaves of a cabbage, it will be possible to include a very fair proportion of the outer leaves in the dried product without rendering it stringy and unpalatable. The presence of a certain amount of leaf in the dried material also tends to improve its appearance. In all our experiments, therefore, a fair quantity of the more tender leaves has been included. Instead of breaking up the head, the trimmed cauliflower has been cut into thin slices. This procedure makes preparation easier when working on the full scale.

Here, too, the question of blanching arises. In our first experiments on cauliflower the material was dried without blanching. Drying took 23 hr. and the yield was 10·6 %. The fresh material contained 79·4 mg. ascorbic acid/100 g.; the dried material 289·8 mg./100 g. The dried material was brownish but absorbed water well, regaining a close approximation to its original appearance. Because of the protracted drying, blanching was tried to ascertain if killing the tissue would speed up drying. Blanched material was found to dry in 11 hr. and gave a yield of 10·4 %. The marked shortening in the time of drying decided us to make blanching the regular practice. Experiments soon showed that the optimum period for blanching was 4 min. exposure to live steam; a shorter exposure did not suffice to kill the tissue, while longer exposure tended to cook the material and cause a loss of soluble constituents.

In the earlier experiments no trouble was encountered in drying, a temperature of 55° being used. A demand, however, came for the production of a large quantity of dried cauliflower and the trays in the drier were rather heavily loaded. Drying was most uneven and where it was prolonged a bad-smelling slime developed on the moist surfaces. A large motile bacterium was isolated

from the slime in almost pure culture by Dr R. M. Nattrass, Senior Plant Pathologist at these laboratories, to whom our thanks are due. At first it was thought that the organism must of necessity be thermophilic, but it developed rapidly at room temperature, both on artificial medium and on potato. Apparently, although the temperature of the air current in the drier was 55°, this temperature was not attained on the attacked surface. Perhaps rapid evaporation from contiguous surfaces had prevented the temperature from rising. Raising the temperature in the drier to 65–70° prevented bacterial action as long as the rate of loading of the trays was not excessive. As soon as the trays were too heavily loaded, even at these temperatures, some of the cut surfaces were shielded from the air current and on the wet surfaces the bacterium rapidly developed.

Before cooking the dried cauliflower it was found necessary to soak for at least 30 min., preferably longer. If soaking were curtailed, the tissue did not absorb water properly and the cooked material had a most unappetizing appearance. It was also tough. With full soaking, the cooked material closely resembled fresh cauliflower in appearance. Although the plain dried material gave on cooking a product of excellent appearance, the blanched material was even better. The flavour too, was perfect.

As far as ascorbic acid is concerned results are not quite so satisfactory. There is a heavy loss of ascorbic acid, probably occurring during the period of soaking. Results of two typical experiments are given in Table 7.

Table 7

	Plain dried mg./100 g	Blanched and dried mg./100 g.
Fresh cauliflower	79·4	71·3
Dried cauliflower	289·8	462·0
Cooked after soaking:		
Solid	17·3	64·2
Cooking water	20·7	62·1

Probably the heavy loss in the cooking of the unblanched material is due to the action of oxidases during the soaking.

Carrots. Although it is well known that the vitamin C content of carrots is comparatively low (in our experiments this fluctuated about 9 mg./100 g.) they have been included in the vegetables to be dried on account of their high food value and value as a source of other vitamins.

The distribution of ascorbic acid in the carrot was investigated. Tips contained 8·6 mg./100 g., the middle portion 8·6 mg., and the butt end, after discarding the absolute butt, 11·6 mg. This was perhaps to be expected as the butts are often coloured green to the depth of 1 cm.

The preparation of carrots for drying is very simple. With young carrots there is even no need for peeling but with more mature material this is desirable. The cleaned and topped carrots are either sliced into circles or cut into strips. Drying is done at 150° F. and is complete in 8–10 hr. The yields of dry material obtained in a number of experiments lay between 13 and 14 %. Here, too, the question of blanching was studied. The sole effect of blanching was to give a lower yield, in one experiment from 13·3 to 12·1 %, while on cooking the flavour of the blanched material was flat.

Green beans. In the preparation of beans for cooking the waste is comparatively small so it did not appear necessary to investigate the distribution of ascorbic acid, but the variation with age was studied. Very young beans had a

content of 19–19·6 mg./100 g. More mature pods, in which the strings were beginning to form, contained 24·7–26 mg./100 g.; older beans 24·1–26·2 mg./100 g.; very old beans had a content of 27·7–29·4 mg./100 g. Thus, in maturation there is not a sufficient increase in ascorbic acid to make it worth while processing old beans which would require a great deal of manual labour in 'stringing'. Beans up to the stage where the strings become evident will therefore be used for the production of dried vegetables. Such beans will need cutting into strips only. The drying temperature to be employed is 130° F. The yields of dried beans obtained in several experiments varied from 10·6 to 12·5 %. Here, too, the effect of blanching was investigated, a steaming time of 3 min. being used. Blanching depressed the yield of dry material by 1·5 %. The ascorbic acid content of blanched dried beans was 74·5 mg./100 g., while that of a parallel lot dried without blanching was 95·1 mg. The colour of the dried blanched beans was a faded yellow-green, whereas that of the unblanched dried beans was a good green with a silvery tint. On cooking, the difference in colour was even more marked; the blanched beans were tough, hard and of a brown colour, while the unblanched were quite normal in texture and of a green colour. The flavour of the unblanched was distinctly better. Unexpectedly, blanching also had a very deleterious effect on the ascorbic acid content. A well-mixed lot of fresh beans was divided into two; one-half after cutting was blanched and dried, the other dried without blanching. The dried products were carefully sampled and cooked in duplicate quantities with and without pre-soaking. The results of this experiment are given in Table 8.

Table 8. *Effect of blanching and pre-soaking on ascorbic acid content of cooked dried green beans*

	Blanched mg./100 g.	Unblanched mg./100 g.
(1) Unsoaked:		
(*a*) Solid	35·9	53·2
Cooking water	30·9	74·2
(*b*) Solid	26·0	32·2
Cooking water	31·6	61·8
(2) Soaked:		
(*a*) Solid	13·4	13·0
Cooking water	13·0	11·1
(*b*) Solid	14·8	9·9
Cooking water	14·2	6·2

A further test was made on the palatability of cooked beans with and without pre-soaking and it was found that there was no difference in flavour; the recommendation has therefore been made, as with cabbage, that dried green beans should not be pre-soaked but dropped straight into boiling water.

Potatoes. Probably the best known method of drying potatoes is the production of potato flakes, but it was felt by all concerned that potatoes in this form would very soon become monotonous as an article of diet. What is really required is some form which can be prepared in different ways. Plain sliced potatoes do not boil up well but fairly massive chips can be both boiled and fried, and this form has therefore been employed in all the experiments.

Because of the danger of discoloration after cutting, it was considered essential that potatoes should be blanched. In course of the experiments to determine the optimum time of blanching some more unexpected results were obtained. Blanching for a period of 60 sec. in live steam sufficed to prevent discoloration

and the dried material was quite attractive; it was fairly white and the individual chips were opaque. However, on attempting to boil this dried material, the real problem was encountered. Even after soaking overnight and cooking for 1 hr. the material did not soften. Increasing blanching periods were tried but, until the time of 3 min. was reached, this trouble continuously occurred.

Occasionally, the material after being steamed for less than 3 min. tended to turn brown, but this did not occur in that blanched for 3 min., unless a long time elapsed between steaming and entry into the drier. Drying was fairly fast, 8½–10 hr., at a loading of 2–3 lb. per sq. ft. When properly treated, the dried material was translucent and had a pale yellow colour. The sides of the chips were deeply concave.

In the first experiments on cooking, the dried chips were soaked overnight, absorbing approximately double their own weight of water. Cooking was complete after 25 min. The colour of the cooked material was perfect and whether eaten whole or mashed the flavour was practically indistinguishable from that of the fresh potato. In one or two cases the opinion was expressed that the flavour was rather more pronounced than that of the fresh potato. Potatoes soaked overnight were also fried, giving a product indistinguishable in every respect from fried fresh potatoes. It was later ascertained that the minimum soaking required to obtain a passable product is 2 hr.; this is rather too short for a perfect product as the chips do not soften fully.

The dried material shows no tendency whatsoever to absorb water from a wet atmosphere. A sample has been left standing about in the laboratories in an open bag for 7 months, the last of which was very wet. The dried chips were as hard as they were on the day they were prepared and cooked up perfectly, whether boiled or fried. Although during the experimental work the ascorbic acid content of the dried material was not considered, an assay was made on the 7 months-old material. The ascorbic acid content was 49 mg./100 g., so dried potatoes can make a useful contribution to the vitamin C requirements.

Summary

Studies were made of the ascorbic acid content and palatability of dried vegetables, including cabbages, cauliflowers, beans, carrots and potatoes.

Details of methods used are described. Potatoes and cauliflowers should be blanched before drying, while carrots, green beans and cabbages should be dried without previous blanching.

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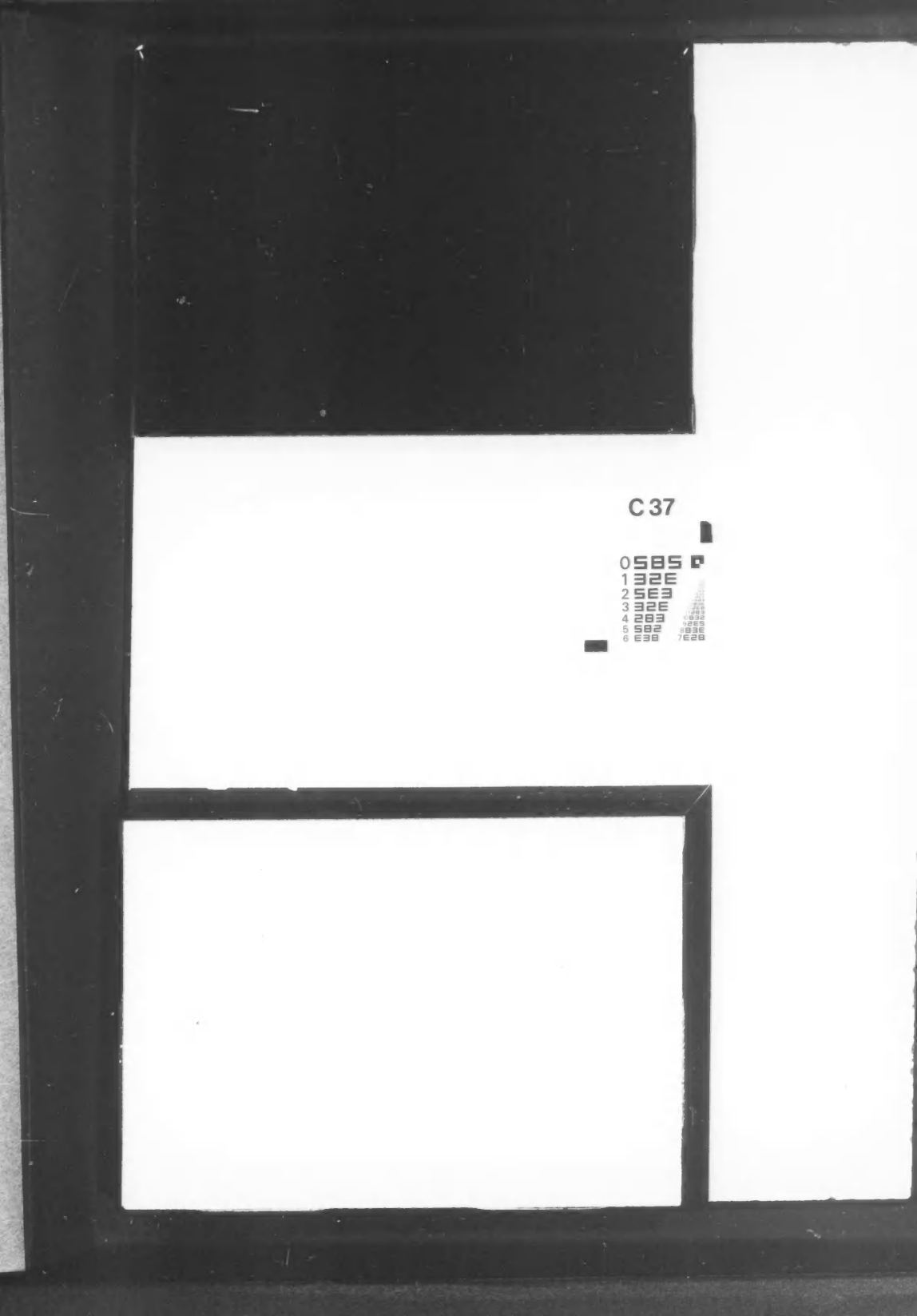
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